REMARKS

Claims 1, 2, 4, 6-9, and 12-24 are pending in this application. Claims 2, 4, 8, 9, and 12-20 have been withdrawn as being directed to a non-elected invention. Claims 1, 6, 7, and 21-24 are rejected under 35 U.S.C. § 103(a) for obviousness over Deboer (U.S. Patent No. 5,633,076; hereinafter "Deboer"), Clark (U.S. Patent No. 5,322,775; hereinafter "Clark"), or Lubon (U.S. Patent No. 5,831,141; hereinafter "Lubon") in view of Morinaga et al. (PNAS 80:4604-4608; 1983; hereinafter "Morinaga") and Bennett (Breast Cancer Res. Treatment 45:169-179, 1997; hereinafter "Bennett"). By this reply, Applicants cancel claims 2, 4, 8, 9, and 12-20, amend claims 6, 21, and 23, add new claims 25-27, and address each of the Examiner's rejections.

Support for the Amendment

Support for the amendment to claims 6, 21, and 23 is found in the specification at, e.g., page 5, lines 8-11, and page 9, lines 12-17. Support for new claims 25-27 is found in the specification at, e.g., page 12, lines 25-26. No new matter is added by the amendment.

Rejections under 35 U.S.C. § 103

Claims 1, 6, 7, and 21-24 are rejected under 35 U.S.C. § 103(a) for obviousness over Deboer, Clark, or Lubon in view of Morinaga and Bennett. The Office states:

> Each of DeBoer, Clark and Lubon taught making transgenic mammals that produce and secrete a recombinant protein of interest into the milk of said mammal. As set forth in the rejection of record, the vast array of mammals used and proteins produced by said mammals renders obvious the method of making a mammal to express any protein of interest and to collect it in the milk given that Morinaga provided the additional teachings and motivation to apply the methods of each of DeBoer, Clark and Lubon to produce rHuAFP in the milk of mammals. Each of DeBoer, Clark and Lubon taught collecting and purifying their respective recombinant proteins, rendering it obvious to do so to rHuAFP as well. (Final Office Action, p. 3.)

Applicants respectfully disagree.

A claimed invention is unpatentable if the differences between it and the prior art are such that the claimed subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. *See* 35

U.S.C. § 103(a) (2003). Correspondingly, the conclusion regarding obviousness of a claimed invention is based upon the following four factual inquiries: (1) the scope and content of the prior art; (2) the differences between the claims and the prior art; (3) the level of ordinary skill in the pertinent art; and (4) secondary considerations of nonobviousness (*e.g.*, commercial success, long-felt but unsolved needs, failure of others). *See McNeil-PPC, Inc. v. L. Perrigo Co.*, 67 U.S.P.Q.2D (BNA) 1649, 337 F.3d 1362, 1368 (Fed. Cir. 2003) (citing *Graham v. John Deere Co.*, 383 U.S. 1, 17-18 (1966)); *Brown & Williamson Tobacco Corp. v. Philip Morris Inc.*, 229 F.3d 1120, 1124 (Fed. Cir. 2000) (same); *Ex Parte Crinion*, No. 2001-0210, 2002 WL 31257831, at *2 (Bd. Pat. App. & Interf. 2001) (same); MPEP § 2141.

"A prima facie case of obviousness may be rebutted by showing that the art, in any material respect, teaches away from the claimed invention. *In re Geisler*, 116 F.3d 1465, 1471, 43 USPQ2d 1362, 1366 (Fed. Cir. 1997)." (M.P.E.P. § 2144.05(III); emphasis added.) *See also KSR International Co. v. Teleflex Inc.*, 127 S. Ct. 1727, 1739–40 (explaining that when the prior art teaches away from a combination, that combination is more likely to be nonobvious). A reference teaches away when a skilled artisan, upon reading the reference, would be led on a divergent path from the one taken by the Applicants. Although "any need or problem known in the field of endeavor at the time of invention and addressed by the [present application] can provide a reason for combining the elements in the manner claimed" (*see KSR*, 127 S. Ct. at 1742), it is necessary for the Office to establish a sufficient basis for concluding that one skilled in the art would combine the reference teachings to yield the claimed invention. *See In re Icon Health and Fitness, Inc.*, 2007 U.S. App. LEXIS 18244, at 13-14.

Present claims 1, 6, 7, and 21-27 are directed to a substantially pure nucleic acid molecule that includes: (i) a nucleic acid sequence encoding recombinant human alpha-fetoprotein (rHuAFP), (ii) a milk-specific promoter that is operably linked to the rHuAFP-encoding sequence, and (iii) a leader sequence encoding a protein secretory signal that enables secretion of rHuAFP by milk-producing cells into the milk of a mammal (Claim 1 and 25), a non-human transgenic mammal (e.g., a goat, cow, sheep, or pig) having the nucleic acid molecule described above which is inserted into the genome of the mammal, which promotes expression of biologically active rHuAFP in mammary epithelial cells of the mammal (Claims 21, 22, and 27),

a method of preparing biologically active rHuAFP from the milk of the transgenic mammal described above (Claims 23 and 24), and milk from the non-human mammal described above that contains biologically active rHuAFP (Claims 6, 7, and 26). The Office admits that none of DeBoer, Clark, or Lubon teaches each and every limitation of the instant claims. In particular, the Office states that "[t]he only elements lacking from...[DeBoer, Clark, and Lubon], alone or in combination, is the sequence encoding AFP and motivation to use the sequence in the techniques of any of DeBoer, Clark or Lubon" (see pp. 6-7 of Office Action dated October 31, 2005; emphasis added). Therefore, to remedy the deficiencies of DeBoer, Clark, and Lubon, the Office cites Morinaga and Bennett, stating that "Morinaga provided the additional teachings and motivation to apply the methods of each of DeBoer, Clark and Lubon to produce rHuAFP in the milk of mammals," while "Bennett supports a motivation to make recombinant AFP...[and] merely exemplifies the interest in producing large quantities of AFP" (Office Action dated January 30, 2007, pp. 3 and 4, respectively). For the reasons given below, one of skill in the art would not combine DeBoer, Clark, or Lubon with Morinaga and Bennet to yield the invention of present claims 1, 6, 7, and 21-27. Thus, the rejection of claims 1, 6, 7, and 21-24 for obviousness over these references should be withdrawn.

The Scope and Content of the Prior Art

Deboer discloses a method of producing transgenic non-human animals (i.e., non-human primates, mice, cattle, dogs, pigs, and sheep) that produce recombinant polypeptides (i.e., exogenous proteins: human milk proteins, such as lactoferrin, lysozyme, secreted immunoglobulins, lactalbumin, bile salt-stimulated lipase, human serum proteins, such as albumin, immunoglobulins, Factor VIII, Factor IX, protein C, and industrial enzymes, such as proteases, lipases, chitinases, and liginases, and endogenous proteins: bovine milk proteins, such as αS1, αS2, β- and κ-casein, β-lactoglobulin lactoferrin, lysozyme, cholesterol hydrolase, serum proteins, such as serum albumin, and proteinaceous hormones, such as growth hormones) in the milk of the female transgenic animals (see, e.g., col. 6, line 40, through col. 7, line 35). Deboer exemplifies the production of recombinant polypeptides in transgenic cows and mice. Deboer, despite listing many proteins to be expressed in the described system, fails to make mention of

rHuAFP.

Clark discloses a method of producing a recombinant polypeptide (i.e., peptide hormones, blood coagulation factors (e.g., factors VIII and IX or subunits thereof, blood proteins, e.g., beta-globin, and serum proteins, e.g., alpha$_1$-antitrypsin) proteins for foodstuffs, including natural or altered milk proteins of the host mammal, or enzymes) in the milk of a transgenic non-human mammal (i.e., mice, sheep, goats, pigs, and cattle; see, e.g., col. 1, line 39, through col. 2, line 11, col. 3, lines 58-66, and col. 18, line 25, through col. 19, line 11). Clark exemplifies the production of recombinant alpha$_1$-antitrypsin and Factor XI in transgenic sheep and recombinant beta lactoglobulin in mice. Clark also fails to mention rHuAFP.

Lubon discloses the production of recombinant human protein C in the milk of a transgenic animal (i.e., a mouse, a rat, a rabbit, a pig, a sheep, a goat, or a cow; see, e.g., col. 3, line 39, through col. 5, line 55, and col. 7, lines 34-38). Lubon exemplifies the production of recombinant human protein C in transgenic mice and pigs. Lubon also fails to mention rHuAFP.

As is acknowledged by the Office, none of Deboer, Clark, or Lubon, either alone or in combination, teaches or suggests a rHuAFP-encoding nucleic acid construct, a transgenic non-human mammal that expresses and secretes rHuAFP into its milk, methods of producing rHuAFP by using a transgenic non-human mammal to express and secrete rHuAFP into its milk, or milk of a transgenic non-human mammal that contains rHuAFP. To remedy the deficiencies of Deboer, Clark, and Lubon, the Office cites Morinaga and Bennett, which the Office asserts identifies HuAFP as a protein of interest (see Office Action dated October 31, 2005, p. 7). Applicants have previously pointed out that Morinaga merely discloses the nucleic acid and predicted amino acid sequence of human AFP, but does not suggest the expression of rHuAFP in a transgenic non-human mammal under the control of a milk-specific promoter or the secretion of rHuAFP in the milk of that mammal based on the presence of a leader sequence, as is taught in the present specification and recited in present claims 1, 6, 7, and 21-27. Furthermore, Morinaga fails to provide any motivation to express human AFP using recombinant means of any sort.

Bennett discloses the expression of rHuAFP using an *E. coli* expression system (see, e.g., the Abstract). Bennett concludes that the "[a]vailability of large quantities of homogeneous, biologically active recombinant human AFP will facilitate further studies of structure/function,

mechanism, and therapeutic potential of this agent as a regulator of breast cancer growth" (Abstract). Bennett, like Morinaga, fails to teach or suggest a nucleic acid molecule containing a nucleic acid sequence encoding rHuAFP, a milk-specific promoter, and a leader sequence. Bennett also fails to teach or suggest the expression and secretion of biologically active rHuAFP in the milk of a transgenic non-human mammal; Bennett is limited solely to the expression of rHuAFP in *E. coli*. Thus, nowhere does Morinaga or Bennett provide any teaching or suggestion to express rHuAFP in a transgenic non-human mammal, much less the production of a transgenic mammal capable of secreting rHuAFP into its milk.

The Prior Art Teaches Away from the Expression of rHuAFP in the Milk of a Transgenic Mammal

Even if Morinaga and Bennett could be read to direct the skilled artisan to express rHuAFP in the milk of a transgenic mammal according to the methods of Deboer, Clark, or Lubon, which they do not, publications available prior to Applicant's filing date direct the skilled artisan **away** from the expression of rHuAFP in milk. Milk is known to contain an abundant amount of free fatty acids (FFAs), including mono- and poly-unsaturated acids (see Table 3 from "Compositions of Foods; Dairy and Egg Products"; Agricultural Handbook No. 8-1, Agricultural Research Service; a copy of which is enclosed as Exhibit A).

With respect to this point, Applicants first direct the Office to Vallette et al., which teaches that "an unsaturated fatty acid environment induces conformational changes in [rat and human] AFP which may modulate the endocrine and immune functions of this protein" (see Abstract, Biochim. Biophys. Acta 997:302-312, 1989; a copy of which is enclosed as Exhibit B). Vallette et al. further states that "UFA [unsaturated fatty acids] can inhibit estrogen binding to both mouse and rat AFP. Inhibition is dose-dependent and varies with the degree of fatty acid unsaturation, polyunsaturated fatty acids such as arachidonic acid and docosahexaenoic acids are the most efficient" (see page 302). Furthermore, Vallette teaches that "incubation [of rodent and human AFPs] with unsaturated fatty acids caused major changes in the immunological behaviour of AFP. The unsaturated fatty acid-loaded AFP (L-AFP) from mouse, rat and human ...were considerably less immunoreactive towards specific polyclonal anti-N-AFP antibodies" (see p.

307). Finally, Vallette states that "unsaturated fatty acids appear to affect the conformation of AFP, specifically influencing its estrogen-binding activity" (see p. 310).

Similarly, Haourigui et al. teaches that FFAs "play a major role in the transfer of hormonal information...[and] "induce subtle specific changes in the binding of hormones to several plasma proteins, including murine α-fetoprotein (AFP)" (page 157; Biochimica et Biophysica Acta 1125:157-165, 1992; a copy of which is enclosed as Exhibit C). In addition, Parmelee et al. teaches that HuAFP "has been found to contain a variety of fatty acids, some of a type not noted in adult albumin or seen only in trace amounts" (J. Biol. Chem. 253:2114-2119, 1978; a copy of which is provided as Exhibit D). In particular, Table 1 of Parmelee et al. shows that AFP is capable of binding several different types of FFAs, including many of the monounsaturated fatty acids and polyunsaturated acids present in mammal milk (page 2117). Parmelee further states "[w]e have also confirmed that human AFP binds the [polyunsaturated fatty acids] with high affinity" (Abstract).

These studies clearly show that FFAs induce conformational changes in HuAFP that inhibit its biological activity, such as estrogen binding, and that the inhibition is dose dependent, varies with the degree of unsaturation of the FFAs, and results from a FFA-induced conformational change in the HuAFP, which is reflected in alterations in the immunological behavior of this protein. In fact, Applicants confirmed that HuAFP secreted into the milk of a transgenic goat does indeed bind FFAs present therein. As shown in Table 1 (enclosed) HuAFP obtained from transgenic goat milk binds several types of FFAs, including, e.g., palmitic acid, linoleic acid, oleic acid, strearic acid, and heptadecanoic acid.

Thus, given the time and expense required to produce a transgenic mammal, one skilled in the art, having knowledge of, e.g., Vallete et al., Haourigui et al., and Parmelee et al., all of which teach that HuAFP, when exposed to an environment rich in FFAs, will bind to FFAs which induce changes in the conformation and biological activity of HuAFP, would not seek to produce biologically active rHuAFP in the milk of a transgenic mammal. These references clearly direct the skilled artisan **away** from the expression of rHuAFP in the milk of a transgenic mammal. Because Vallete et al., Haourigui et al., and Parmelee et al., among others, materially teach away from the use of Applicant's claimed compositions and methods (see *In re Geisler*,

supra, and *KSR International Co. v. Teleflex Inc.*, *supra*), the skilled artisan would have no motivation to combine Deboer, Clark, or Lubon with Morinaga and Bennett to yield the invention of present claims 1, 6, 7, and 21-27. For this reason, the rejection of claims 1, 6, 7, and 21-24 for obviousness over Deboer, Clark, or Lubon in combination with Morinaga and Bennett should be withdrawn.

There is no Reasonable Expectation that Expressing rHuAFP in the Milk of a Transgenic Mammal would be Successful

Even if the Office disagrees that the prior art publications discussed by Applicants above would lead the skilled artisan away from the expression of rHuAFP in the milk of a transgenic mammal according to the methods of Deboer, Clark, or Lubon, there is still no reasonable basis for the Office to conclude that any of "Deboer, Clark, and Lubon render obvious the use of the claimed mammalian system to make any recombinant protein with a reasonable expectation of success" (Final Office Action dated January 30, 2007, p. 7).

The *KSR* Court recognized that "[w]hen there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill has good reason to pursue the known options within his or her technical grasp" (*KSR*, 127 S. Ct. at 1732). Under these circumstances, "the fact that a combination was obvious to try might show that it was obvious under § 103." *Id.* That is not the case here.

As is discussed above, none of Deboer, Clark, or Lubon teaches or suggests the expression of rHuAFP in the milk of a transgenic mammal.[1] More than this, though, each of Deboer, Clark, and Lubon fail to provide any reasonable expectation that rHuAFP could be successfully expressed in the milk of a transgenic mammal in a biologically active form. The only serum proteins that Deboer suggests can be expressed in the milk of a transgenic mammal are serum albumin, immunoglobulins, Factor VIII, Factor IX, and human protein C, while Clark and Lubon only suggest the expression of alpha$_1$-antitrypsin and protein C, respectively, in the

[1] The Office acknowledges that it relies on Morinaga for its disclosure of the sequence of HuAFP (see Office Action dated March 22, 2005, p. 12), while Bennett is cited because it provides HuAFP as a protein of interest (see Office Action dated January 30, 2007, p. 4). Because the Office does not rely on Morinaga and Bennett for a teaching or suggestion to produce a transgenic mammal capable of expressing rHuAFP, these references are not discussed here.

milk of a transgenic mammal. None of Deboer, Clark, or Lubon, though, provides any evidence that these or any other serum proteins are expressed in the milk of a transgenic mammal in a biologically active form. In fact, Lubon confirms that the expression of recombinant proteins, in particular protein C, in a transgenic mammal is highly unpredictable and may result in the expression of a significant amount of biologically *inactive* protein (i.e., ~60-70% of the expressed protein C is inactive), stating:

> The expression of therapeutic proteins by recombinant DNA technology is an attractive alternative to plasma production of protein C, in that it eliminates the risk of potential contamination with blood-borne viruses and theoretically provides an unlimited supply of product. **But the complexity of the post-translational modifications, as discussed above, has rendered problematic the production of commercially useable amounts of suitably active protein C by expression in a heterologous host.**
>
> **In fact, it has not been possible to produce vitamin K-dependent proteins like protein C at sufficiently high levels in an active form, despite efforts to do so using a variety of expression systems.** See Grinnell et al. in Volume 11 of ADVANCES IN APPLIED BIOTECHNOLOGY SERIES, Chapter 3 (Gulf Publishing Co.). **In particular, any prospect for expressing protein C in mammary glands of a transgenic animal and secreting the protein into milk, see, e.g., U.S. Pat. No. 4,873,316 (1989), is clouded by the fact that protein C is normally synthesized in the liver.** Even HepG2 cell lines derived from human liver produce aberrant forms of protein C. Marlar & Fair (1985).
>
> **In this regard, it has been observed that a mouse mammary epithelial cell line (C-127) transfected with a bovine papilloma virus (BPV) vector bearing the cDNA for human protein C expressed protein C that was only 30-40% active.** Further analysis revealed that the protein C contained diminished levels of γ-carboxyglutamic acid and little, if any, β-hydroxyaspartic acid. Suttie et al., Thrombosis Res. 44: 129 (1986). **These experiments indicate that mouse mammary epithelial cells cannot perform all of the post-translational modifications necessary for obtaining suitably active protein C, which in turn casts doubt on the likelihood of obtaining such protein C from the milk of a transgenic mammal.** (See Col. 3, lines 3-36; emphasis added.)

Lubon purports to solve the problems associated with the expression of protein C in the milk of a transgenic mammal, but fails to explain how one skilled in the art would overcome the issue of unpredictability with respect to any other recombinant protein expressed in the milk of a transgenic mammal. Neither Deboer nor Clark remedies this deficiency. Thus, none of Deboer, Clark, or Lubon provides any reasonable basis to conclude that any serum protein, and certainly

not rHuAFP, can be expressed in the milk of a transgenic mammal in a biologically active form. For this reason, Deboer, Clark, and Lubon fail to provide the skilled artisan with any reasonable expectation of success with respect to the expression of biologically active rHuAFP in the milk of a transgenic mammal.

Moreover, as is discussed above, prior to Applicants' filing date the skilled artisan was well aware that the binding of FFAs to AFP induces a conformational change in AFP that inhibits its biological activity (see Vallete et al., Haourigui et al., and Parmelee et al., discussed above). Because FFAs are present in significant amounts in the milk of mammals, one skilled in the art, having knowledge of Vallete et al., Haourigui et al., and Parmelee et al., and absent evidence to the contrary, would have no reasonable expectation that the expression of rHuAFP in the milk of a transgenic mammal would yield biologically active rHuAFP; nothing in Deboer, Clark, Lubon, Morinaga, or Bennett changes this conclusion. Thus, Applicants respectfully submit that, for this reason as well, the rejection of claims 1, 6, 7, and 21-24 under 35 U.S.C. § 103(a) for obviousness over Deboer, Clark, or Lubon in combination with Morinaga and Bennett should be withdrawn.

CONCLUSION

Applicants submit that present claims 1, 6, 7, and 21-27 are in condition for allowance, and such action is respectfully requested.

Enclosed is a petition to extend the period for replying for four months, to and including November 13, 2007, and a check for the fee required under 37 C.F.R. § 1.17(a).

If there are any other charges, or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

TODD ARMSTRONG, Ph.D.
Reg. No. 54,590

Date: October 31, 2007

for Paul T. Clark
Reg. No. 30,162

Clark & Elbing LLP
101 Federal Street
Boston, MA 02110
Telephone: 617-428-0200
Facsimile: 617-428-7045

Exhibit A

TABLE 3. COMPARISON OF COW, GOAT, AND HUMAN MILK/100 gms			
FATTY ACID (gm)	COW	GOAT	HUMAN
Saturated			
Total	2.80	2.67	2.01
C4:0	.11	.13	—
C6:0	.06	.09	—
C8:0	.04	.10	—
C10:0	.08	.26	.06
C12:0	.09	.12	.26
C14:0	.34	.32	.32
C16:0	.88	.91	.92
C18:0	.40	.44	.29
Monounsaturated			
Total	.96	1.11	1.66
C16:1	.08	.08	.13
C18:1	.84	.98	1.48
C20:1	trace	—	.04
C22:1	trace	—	trace
Polyunsaturated			
Total	.12	.15	.50
C18:2	.08	.11	.37
C18:3	.05	.04	.05
C18:4	trace	—	—
C20:4	trace	—	.03
C20:5	trace	—	trace
C22:5	trace	—	trace
C22:6	trace	—	trace

Source:
Adapted from "Composition of Foods; Dairy and Egg Products", Agricultural Handbook No. 8-1, Agricultural Research Service, Washington, D.C.; USDA, 1976.

Exhibit B

Biochimica et Biophysica Acta, 997 (1989) 302–312
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BBAPRO 33423

Conformational changes in rodent and human α-fetoprotein: influence of fatty acids

Geneviève Vallette, Roger Vranckx, Marie-Elise Martin, Claudine Benassayag and Emmanuel A. Nunez

INSERM U.224, affiliée au CNRS, Laboratoire de Biochimie, Faculté de Médecine Xavier Bichat, Paris (France)

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Key words: α-Fetoprotein; Fatty acid; Protein conformation; Estrogen binding; Immunodetection

Binding, spectral and immunological studies were performed to demonstrate the conformational changes in rodent and human α-fetoprotein (AFP) induced by a free fatty acid environment. Scatchard analysis of estradiol (E2) binding to purified rat AFP indicated that unsaturated fatty acids changed the number of binding E2 sites and the apparent E2 equilibrium dissociation constant which varied non-linearly with docosahexaenoic acid concentration. UV spectral analysis of rodent and human AFPs showed that the absorbance minimum of AFP incubated with unsaturated fatty acid (L-AFP) was red-shifted, broadened and less pronounced than that of purified native AFP (N-AFP). Immunochemical studies with specific polyclonal antibodies to purified rodent and human AFPs (N-AFP antibodies) showed that these proteins lost immunoreactivity after incubation with unsaturated fatty acid. N-AFP antibodies recognized fewer epitopes on L-AFP than on N-AFP, whatever the species. Specific anti-rat L-AFP antibodies were used to demonstrate specific epitopes on rat L-AFP. Rat L-AFP antibodies did not recognize rat N-AFP. Saturated fatty acids were without effect on the binding, spectral and immunological properties of rodent and human AFPs. RIA or ELISA values for human AFP from fetal serum, hepatoma serum, and cord serum, were reduced 80, 50 and 5%, respectively, by unsaturated fatty acids. This decrease correlated with the relative percentage of polyunsaturated fatty acid in each biological fluid. Such results indicate that an unsaturated fatty acid environment induces conformational changes in AFP which may modulate the endocrine and immune functions of this protein.

Introduction

Mammalian α-feto-proteins (AFPs) are oncofetal antigens which exhibit a complex molecular heterogeneity involving differences in size, charge, carbohydrate moiety and ligand contents (fatty acids and estrogens) [1–3]. The molecular polymorphism of AFPs is associated with considerable intra- and inter-species functional heterogeneity reflected in its binding [1–3] and immunomodulatory properties [4].

While all mammalian AFPs bind non-esterified fatty acids (NEFAs), especially unsaturated fatty acids (UFA), with high affinity ($K_a = 10^6$–10^7 M^{-1}) [5–8], only rodent AFPs have been shown to bind estrogen [9]. Several studies have shown that the binding of estrogen to rodent AFP is modulated by the endogenous NEFA content of the protein, which in turn depends on its exogenous NEFA environment [5–6,10]. Thus, UFA can inhibit estrogen binding to both mouse and rat AFP. Inhibition is dose-dependent and varies with the degree of fatty acid unsaturation, polyunsaturated fatty acids such as arachidonic and docosahexaenoic acids are the most efficient. Fatty acids (FA) have been reported to have similar effects on the binding properties of other plasma proteins, such as sex steroid-binding protein (SBP) [11] corticosteroid-binding globulin (CBG) [12], and on the binding of thyroid hormones by plasma proteins [13].

The immunoregulatory effects of AFP include the suppression of primary and secondary antibody responses [14], lymphocyte transformation induced by mitogens and allogenic cells [15,16], and Ia macrophage antigen expression [17]. However, other authors were unable to obtain similar results using purified AFP

Abbreviations: AFP, α-fetoprotein; E2, estradiol; E1, estrone; L-AFP, AFP incubated with unsaturated fatty acid; N-AFP, native AFP; NEFA, nonesterified fatty acid; UFA, unsaturated fatty acid; FA, fatty acids; SBP, sex steroid-binding protein; CBG, corticosteroid-binding protein; PBS, phosphate-buffered saline.

Correspondence: E.A. Nunez, INSERM U.224, affiliée au CNRS, Laboratoire de Biochimie, Faculté de Médecine Xavier Bichat, 16, rue Henri Huchard, 75018 Paris, France.

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preparations. Some obtained either stimulatory effects or no response [18,19]. The varying immunomodulatory activities of AFP could be related to the use of different forms of AFP, e.g., AFP isoforms (variable carbohydrate structure) and/or AFP holoforms which may vary according to their variable ligand contents (NEFAs and estrogens) which in turn will depend on the biological source of AFP and the purification method used [20–23].

The relationship between the NEFA-dependent biological activity of AFP and NEFA-induced conformational changes was examined by analysing the physicochemical properties of rat, mouse and human AFPs in the presence of saturated or unsaturated fatty acids. The three parameters studied were the NEFA-induced changes in the E2 binding properties of purified rat AFP, the UV spectral properties of rat and human AFP, and the serological reactivity of mammalian AFP. Special attention was paid to the immunological behaviour of mammalian AFP towards antibodies directed against native (N-AFP) or fatty acid-loaded AFP (L-AFP) and on the differences in human AFP immunological reactivity produced by the lipid environment of various biological fluids (embryo serum, hepatoma serum and cord serum). The results indicate that unsaturated fatty acids induce significant changes in the functional properties, e.g., binding and immunoreactivity of AFP, which are associated with conformational changes in the structure of AFP itself.

Materials and Methods

Chemicals

[6,7-^{3}H]Estradiol (41 Ci/mmol) purchased from Amersham International Ltd. was 98–99% pure and was regularly tested to ensure that level of purity.

Unlabeled compounds

Estradiol (E2) and estrone (E1) were supplied by Roussel Uclaf Research Centre (Romainville, France). The following saturated and unsaturated fatty acids were purchased from Sigma: tetradecanoic acid (14 : 0, myristic acid), hexadecanoic acid (16 : 0, palmitic acid), *cis*-7-hexadecanoic acid (16 : 1, palmitoleic acid), *cis*-9-octadecanoic acid (18 : 1, oleic acid), 9,12-octadecadienoic acid (18 : 2, linoleic acid), 6,9,12-octadecatrienoic acid (18 : 3, linolenic acid), 5,8,11,14-eicosatetraenoic acid (20 : 4, arachidonic acid), 4,7,10,13,16,19-docosahexaenoic acid (22 : 6) and 7,10,13,17-docosatetraenoic acid (22 : 4). NEFA purity was checked by thin-layer chromatography (benzene/methanol/acetic acid, 96 : 4 : 1, v/v). Stock solutions of NEFAs (1 mg/ml) were prepared, using hexane for unsaturated fatty acids and chloroform for saturated fatty acids.

Blood samples

Blood samples were obtained from the umbilical cord of 3–4-month-old human fetuses (after therapeutic abortion) and hepatoma patients. Rats were made hepatotoxic by injection of carbon tetrachloride (CCl_4): 5-week-old Sprague Dawley rats (Charles River, France) were injected, i.p., with 100 μl CCl_4 in 500 μl sesame oil per 100 g body weight. Fetal rat and mouse serum was taken from 19-day rat embryos and 18-day mouse embryos. All blood samples were centrifuged immediately after collection and the serum was stored at $-80\,^\circ$C until used.

Purification of AFPs

AFPs were purified from fetal serum samples by affinity chromatography using specific antisera raised in rabbit against rat and mouse AFP purified by acrylamide gel electrophoresis as previously described [10]. Anti-human monospecific AFP antibodies were a gift from Dr. Keckaert (Lille).

The IgG from these specific antisera were purified by DEAE-Trisacryl chromatography in 0.035 M Tris, 0.025 M NaCl buffer (pH 8.8). The IgG antibody fractions were dialysed against 0.05 M NaCl and coupled to CNBR-activated Sepharose 4B (Pharmacia). AFP was bound at pH 8.6 (0.1 M borate buffer, 0.5 M NaCl). Human AFP was eluted with 0.1 M sodium acetate, 3 M sodium thiocyanate, pH 4; rat and mouse AFPs were eluted with 0.2 M glycine-HCl, 0.5 M NaCl (pH 2.8). The eluted AFP solutions were brought to pH 7 with 0.1 M NaOH. All purified AFP solutions were dialysed for 48 h against distilled water (pH 7) at 4°C. The purified AFPs contained endogenous NEFAs: their concentrations were 4 mol NEFA/mol AFP (human), 5 mol NEFA/mol AFP (rat) and 7 mol NEFA/mol AFP (mouse).

These purified AFP preparations are referred to as native AFPs (N-AFP); the native purified AFPs incubated with exogenous FA are referred to as fatty acid-loaded AFP (L-AFP).

Native purified human and mouse AFPs migrated as broad bands in the α zone on 10% polyacrylamide gel electrophoresis, while rat AFP showed two distinct bands in the α zone. None of the purified AFP preparations produced immunoprecipitin lines against their respective rabbit anti-adult serum (5%) in crossed immunoelectrophoresis, and showed a single immunoprecipitin line against their respective anti-fetal sera (10%).

Antisera

Antibodies were raised in rabbit by the Vaitukaitis method [24].

Monospecific antibodies were raised against the mouse, rat and human N-AFPs, where N-AFP is the purified AFP preparation described above which con-

tains a certain amount of endogenous NEFA. Lipidated AFP (L-AFP) was prepared by incubating 50 μg rat N-AFP in normal saline solution with 500 μg of 22:6, (2 μmol 22:6/nmol AFP, in 0.2 ml) overnight. This L-AFP preparation was used to raise monospecific anti-rat L-AFP antibodies.

Four subcutaneous injections of 20 μg N-AFP or L-AFP in complete Freund's adjuvant were given at 15-day intervals. Immunoglobulins were precipitated from serum at 40% saturated ammonium sulfate and chromatographed on a DEAE-Trisacryl column (0.025 M Tris-HCl, 0.035 M NaCl (pH 8.8)). The N-AFP antibodies and L-AFP antibodies were dialysed against 0.05 M NaCl (pH 7) and stored at −20°C.

Binding studies

Batchwise gel equilibrium (25) at 20°C and Scatchard graphic analysis [26] were used to evaluate the association constant (K_a M^{-1}), dissociation constant ($K_d = 1/K_a$) and the apparent number of binding sites per mol of pure rat AFP. The same techniques were used in competition experiments with free fatty acids. The partition coefficient for E2 was estimated and found to be unaffected by the presence of free fatty acid. The Scatchard analysis and competition studies required a series of tests with fixed amounts of pure rat AFP (58 nM) and [^{3}H]E2 (1.8 nM) plus increasing quantities of non-radioactive identical (1.8 nM to 919 nM, E2 or E1) (Scatchard analysis) or heterologous ligand (7.6 nM to 760 nM, 22:6) (competition analysis). The fatty acid, in organic solvent, were evaporated to dryness under nitrogen, mixed and shaken with buffer overnight at 4°C. The AFP and other reagents were then added. At the concentration used (0.13 to 13 nM 22:6/nM AFP) the solubility was 95%.

Preparation of lipidated AFP

Saturated or unsaturated FA (18:0 or 22:6) were dried under a stream of nitrogen and mixed with purified AFP for immediate testing or stirred with AFP overnight at 4°C.

For HPLC gel filtration studies, 100 μg AFP were incubated with 10–500 μg of 22:6 or 18:0 (i.e., 0.02 to 1 μmol of FA/nmol AFP in 0.1 ml phosphate-buffered saline (PBS) 0.05 M phosphate, 0.15 M NaCl (pH 7.4)).

For immunoelectrophoresis studies, 2.5 μg AFP were incubated with 10–100 μg of 22:6 or 18:0 (i.e., 1–10 μmol FA/nmol AFP) in 50 μl of Laurell immunoelectrophoresis buffer 0.06 M Tris, 0.02 M barbital lactate calcium (pH 8.6).

Unbound FA were separated from bound FA by HPLC gel filtration or with a charcoal-dextran suspension (0.5/0.05 g per 100 ml).

Fatty acid measurements

Fatty acids were extracted from AFP by the method of Chen [27] and from sera as described [28]. They were quantified by gas liquid chromatography [28].

Protein determination

Protein concentrations were measured by the method of Lowry et al. [29].

HPLC and UV spectral studies

Samples were applied to a TSK G3000 SW gel filtration column (7.5 × 600 mm) connected to an LKB 2150 HPLC pump and eluted with PBS at a flow rate of 1 ml/min. Gel filtration separated the fatty acids-AFP complexes from unbound fatty acids. Protein was monitored with an LKB 2140 multiwavelength detector (190–370 nm); the UV spectra of the eluates were integrated at 1-s intervals and stored in a IBM-XT computer connected to the detector. Data compilation was performed with the LKB Wavescan program.

The spectrum of each preparation was the average of all the spectra obtained during the elution of the protein peak.

Serological methods

Native purified AFP samples and the AFP contents of a number of human biological fluids were assayed in the absence or presence of different concentrations of fatty acids by four immunological methods:

(1) Laurell rocket immunoelectrophoresis was performed as described [30].

(2) Rocket line electrophoresis was performed using the method described by Kroll [31]. N-AFP (0.5 μg) or L-AFP (2.5 μg) was mixed in an agarose gel strip (1 cm × 9 cm × 1.2 mm). A series of samples (5 μl) of purified AFP, L-AFP and CCl_4-treated rat serum was placed in wells in the contact gel close to the N-AFP or L-AFP gel strip. The antigens were then run into the agarose gel (8 cm × 9 cm × 1 mm) containing monospecific antiserum (1% anti-N-AFP or 1% anti-L-AFP). The deflection of the AFP precipitin line by the different samples were used to study the cross-reactivity and the amounts of different AFP preparations.

Immunoelectrophoresis was performed at 2 v/cm for 18 h in the Laurell buffer. The gels were dried and stained with Coomassie blue.

(3) Crossed immunoelectrophoresis was performed using the Clark and Freeman modification [32]. Dimension 1: sample proteins (5 μl: 250 ng) were electrophoresed in 1% agarose gels at 8 v/cm, using Laurell buffer at 14°C. Dimension 2: was run overnight with the same buffer at 14°C at 2 v/cm into 1% agarose gel containing 1% antibody. The gels were dried and stained with Coomassie blue.

(4) Immunoassay (RIA-ELISA). Human AFP was quantified with a RIA kit from Abbot Laboratories or CEA (France), and with an ELISA kit from

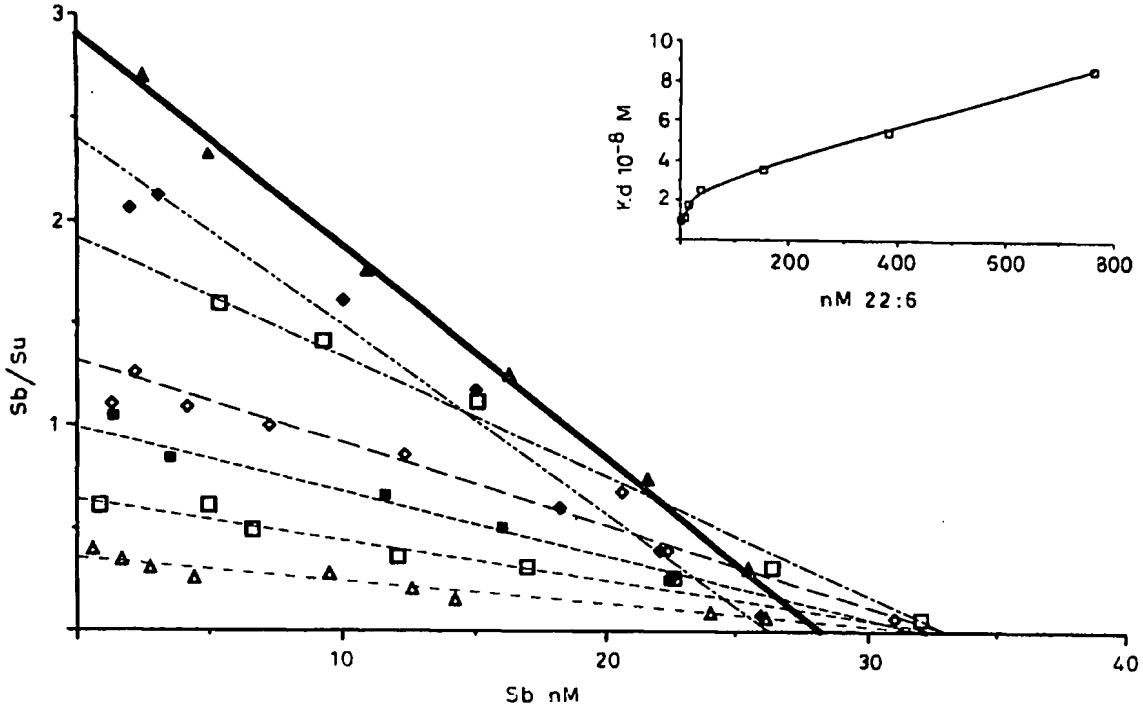


Fig. 1. Scatchard analysis of [^{3}H]E2 binding to purified rat AFP in the presence of docosahexaenoic acid (22:6). 6 μg (58 nM) of purified AFP were incubated 1 h at 20 °C with various concentration of [^{3}H]E2 (1.8–919 nM) in the absence (———▲) or presence of 7.6 nM (— — —◆), 15.2 nM (- — · — -□), 38 nM (— — —◇), 152 nM (· · · · · ·■), 380 nM - - - - - -□), 760 nM (— — —△) of 22:6. Steroid binding was determined by gel equilibration. The straight lines were obtained by linear regression analysis. Sb, bound steroid. Su, unbound steroid. Inset: the apparent equilibrium dissociation constant K_d was plotted against 22:6 concentration.

Boehringer-Mannheim, based on monoclonal anti-human AFP antibodies.

Results

Effects of polyunsaturated fatty acids on estradiol binding purified rat AFP

The binding parameters, association (K_a) and dissociation (K_d) constants and number of binding sites (n), were determined at equilibrium by Scatchard analysis on purified AFP with [^{3}H]E2 in the absence and presence of polyunsaturated FA (22:6).

The binding of E2 was analysed as a function of increasing 22:6 concentration. A low concentration of 22:6 (0.8 10^{-8} M) reduced E2 binding with no change in association constant, but with a slight reduction in

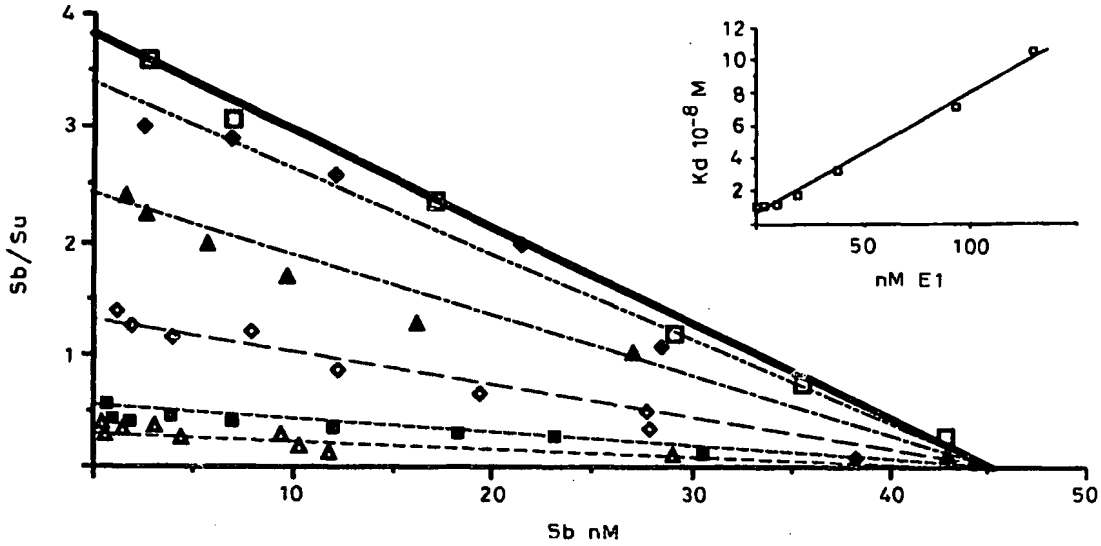


Fig. 2. Scatchard analysis of [^{3}H]E2 binding to purified rat AFP. 6 μg (58 nM) of purified AFP was incubated 1 h at 20 °C with [^{3}H]E2 (1.8–919 nM) in the absence (———□) or presence of 9 nM — — —◆), 18 nM (- — · — -◆), 36 nM (— — —◇), 90 nM (- - - - - -■), 130 nM (- - - - - -△) of non-radioactive estrone (E1). Steroid binding was determined by gel equilibrium. Inset: the apparent equilibrium dissociation constant K_d was plotted against E1 concentration.

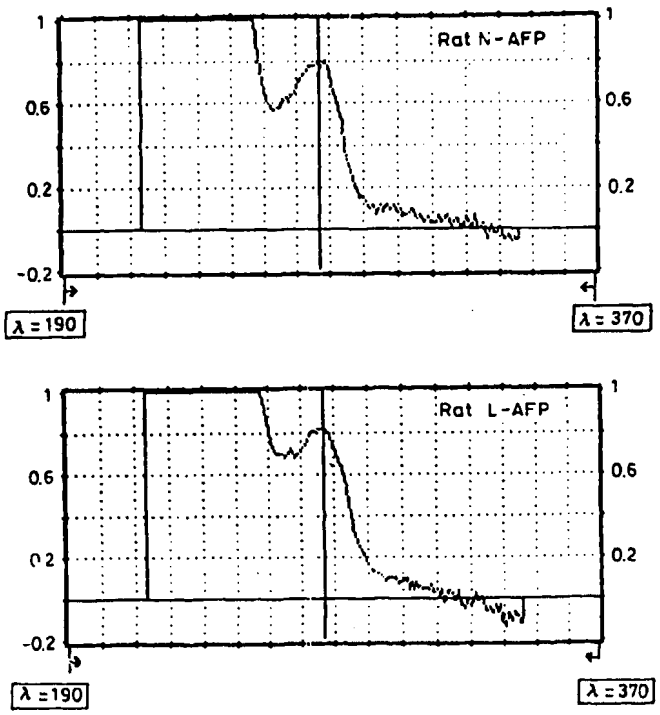


Fig. 3. UV spectra of rat N-AFP and L-AFP. 100 μg of N-AFP or AFP incubated overnight at 4°C in the presence of 0.2 μmol 22:6/nmol AFP (L-AFP) were injected and HPLC chromatographed on a TSKG 3000 SW column in 0.15 M NaCl, 0.05 M phosphate buffer (pH 7.4). Absorption from 190 to 370 nm. The spectrum was the average of all the spectra obtained during elution of the peak. Wavelength of the cursor location is 276 nm.

the number of binding sites ($n = 0.5$ to $n = 0.4$). Higher 22:6 concentrations ($> 1.6 \cdot 10^{-8}$ M) produced a significant dose-dependent decrease in the K_a and a slight increase in the number of binding sites ($n = 0.6$).

When K_d, the apparent equilibrium constant of dissociation for the steroid, was plotted against UFA concentration (inset Fig. 1), the K_d values for E2 increased non-linearly with 22 : 6 concentration. However, Scatchard analysis of E2 binding to AFP in the presence of increasing amounts of unlabelled estrone or estradiol indicated that the K_d for this interaction varied linearly with both increasing concentration of E1 (Fig. 2) and E2 (data not shown).

Effect of fatty acids on AFP spectral properties

The elution profile from HPLC gel filtration column and the UV spectral analysis of the rat N-AFP and L-AFP indicated that both AFP preparations were eluted with the same retention time (14 min and 17 s in our experimental conditions) and showed the same maximum absorbance wavelength ($A_{max} = 276$ nm). However, the absorbance minimum was less pronounced for the L-AFP than for N-AFP (Fig. 3). A plot of the logarithm of the ratio of maximum absorbance at 276 nm over the absorbance at a given wavelength (log $A_{276\ nm}/A_{\lambda nm}$) against wavelength ($\lambda = 245$ to 276 nm), showed that the absorbance minimum (which was 253–254 nm for N-AFP) was red-shifted and broadened to 262 nm for L-AFP (0.2 μmol 22:6/nmol rat AFP) (Fig. 4A). The extent of this shift appears to be FA-dose-dependent. The value of the ratio log $A_{276\ nm}/A_{254\ nm}$ was lower for L-AFP (0.08) than for N-AFP (0.15).

There were similar changes in the absorption spectra of fatty acid-loaded human AFP (L-AFPh) (Fig. 4B). The value of log $A_{276\ nm}/A_{254\ nm}$ dropped in the same dose-dependent manner as for the rat, from 0.15 (N-AFPh) to 0.08 (in the presence of 0.2 μmol 22:6/nmol AFP) and 0.04 (in the presence of 1 μmol 22:6/nmol AFPh). The wavelength 247 nm was taken as the pivotal wavelength at which the ratio plot values for N-AFP were positive and those for L-AFP were negative, for both rat and human AFP.

No spectral changes were seen when either human or rodent AFPs were incubated with saturated fatty acids.

No spectral changes occurred when transferrine, a protein which is not known to bind FA, was incubated

TABLE I

Qualitative and quantitative analysis of non-esterified fatty acids and endogenous content of different biological fluids

NEFA classes		Cord serum		Hepatoma serum		Fetal serum	
Total NEFAs μM:		188		1590		1691	
		(μM)	(%)	(μM)	(%)	(μM)	(%)
Saturated	14:0	6.5		25.6		–	
	16:0	45.6	35.2	27.8	27.25	216.4	35.2
	18:0	39.4		129.3		379.6	
Unsaturated							
mono	16:1	10.9		200		151	
	18:1	47.2	28.8	490	43.3	333.6	28.8
di+tri	18:2	28.8	15.3	346.5	21.8	227.5	13.5
	18:3						
poly	20:4	9.9	5.25	109		130.3	
	22:4	–		–	7.65	–	22.5
	22:6	–		12.5		252.6	

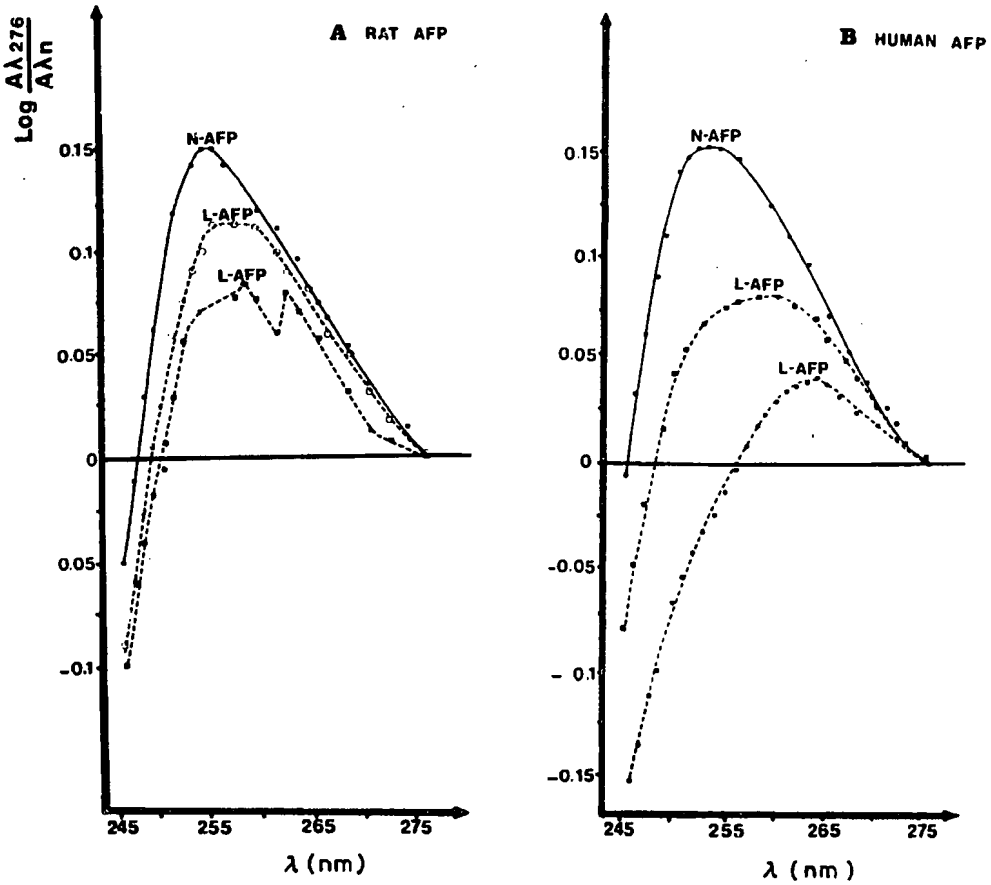


Fig. 4. Plot of the ratio log $A_{276\ nm}/A_{245\ nm\ to\ 276\ nm}$ for N-AFP and L-AFP as a function of wavelength. (A) Rat N-AFP and L-AFP. L-AFP: 100 μg rat AFP were incubated with 50 μg 22:6 (0.1 μmol 22:6/nmol AFP) (○— — —○) and 100 μg 22:6 (0.2 μmol 22:6/nmol AFP) (■— — —■) in 0.1 ml phosphate buffer, overnight at 4°C. (B) Human N-AFP and L-AFP. L-AFP: 100 μg human AFP were incubated with 100 μg 22:6 (0.2 μmol 22:6/nmol AFP (■— — —■) and 500 μg 22:6 (1 μmol 22:6/nmol AFP) (●— — —●) in 0.1 ml phosphate buffer overnight at 4°C.

with increasing concentrations of unsaturated fatty acids (data not shown).

Effects of fatty acids on the immunological properties of rodent and human AFP

The immunoreactivities of N-AFP and L-AFP were first studied with specific polyclonal anti-N-AFP antibodies.

The immunoreactivities of purified rodent and human AFPs were not changed by the presence of saturated fatty acids (e.g., stearic acid), but incubation with unsaturated fatty acids caused major changes in the immunological behaviour of AFP. The unsaturated fatty acid-loaded AFP (L-AFP) from mouse, rat and human migrated slightly faster than the corresponding N-AFPs and were considerably less immunoreactive towards specific polyclonal anti N-AFP antibodies (Fig. 5).

Rocket-line immunoelectrophoresis revealed a loss of L-AFP immunoreactivity with anti-N-AFP antibodies. There was a clear precipitin line between the rat N-AFP of the intermediate gel insert and the N-AFP antiserum in the upper gel (Fig. 6A). The line was deflected to form a well-focused 'rocket' with N-AFP (1st and 2nd wells), but the deflection was very much smaller for the same quantity of L-AFP (3rd and 4th wells).

However, when rat L-AFP was placed in the intermediate gel strip (Fig. 6B), the precipitin line formed with the anti-N-AFP antibody was fuzzy, indicating poor recognition of the L-AFP by polyclonal anti-N-AFP.

The L-AFP rocket was totally fused with the L-AFP line, while the N-AFP rocket showed additional spurs. Thus, some antigenic site(s) were common between N-AFP and L-AFP, but N-AFP had more epitopes than did L-AFP.

When the AFP in a pathological serum (CCl_4-treated rat serum) was tested against an anti-N-AFP antibody (Fig. 6), this AFP deflected the L-AFP line (Fig. 6B) more than the N-AFP line (Fig. 6A). The total fusion of rockets formed by the AFP from CCl_4-treated rat serum with the L-AFP line indicated that it was immunological very similar to L-AFP. Thus the AFP from this pathological serum seems to be different from rat embryo serum AFP.

The differential behaviour of N-AFP and L-AFP led us to prepare specific polyclonal anti-L-AFP antibodies. The immunoreactivity of these antibodies towards both

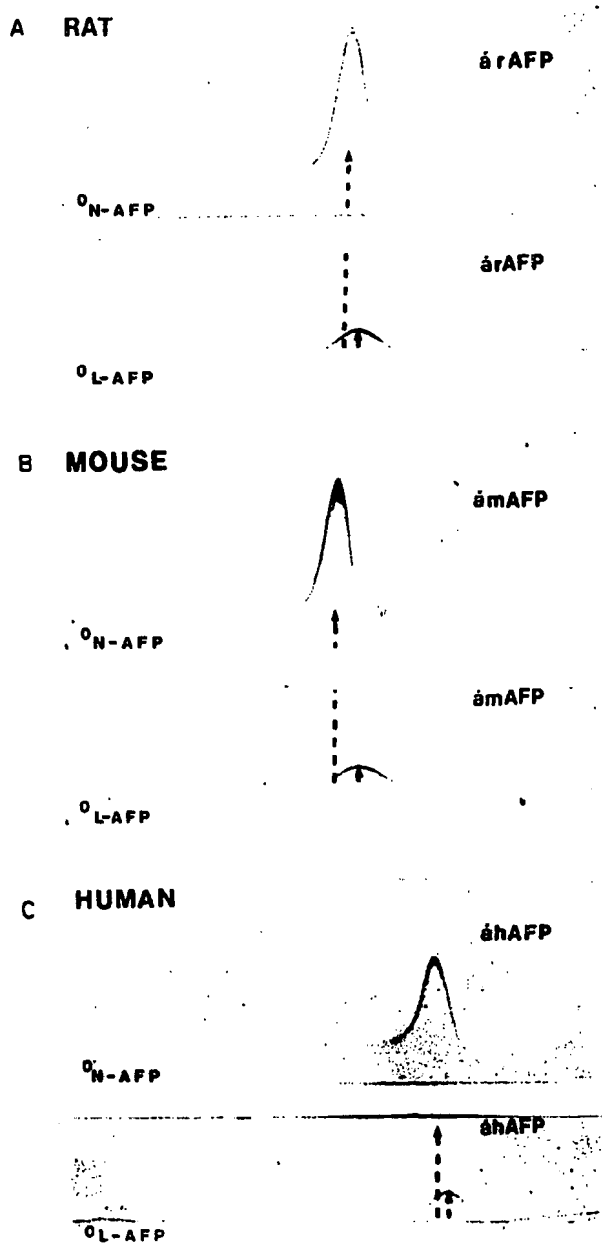


Fig. 5. Crossed immunoelectrophoresis stained with Coomassie blue, with purified rat (A), mouse (B) or human (C) AFP (250 ng) and L-AFPs (250 ng). The L-AFPs were prepared by preincubating AFPs overnight with 22:6 (25 μmol 22:6/nmol AFP). Antiserum 1% = rabbit anti-rat N-AFP (àrAFP), anti-mouse N-AFP (àmAFP), anti-human N-AFP (àhAFP).

native and lipidated AFP preparations were then studied (Fig. 7A, B and C). Rocket-line immunoelectrophoresis with L-AFP in the intermediate gel strip (Fig. 7A) showed a distinct precipitin line with anti-L-AFP antibodies which was clearly deflected by L-AFP, but only fuzzily deflected by N-AFP. By contrast, rocket line immunoelectrophoresis with N-AFP included in the intermediate gel strip (Fig. 7B) showed a fuzzy precipitin line for N-AFP. The L-AFP placed in the wells produced a very small deflection of the fuzzy N-AFP precipitin line, together with an additional clear rocket under the fuzzy precipitin line. These results suggest that the lower part of gel was depleted of N-AFP antibodies and that the well-focused rocket observed with L-AFP is due to the specific recognition of this holoform of AFP by conformation specific-sites of 'monoclonal subclasses' of the polyclonal antibodies raised against L-AFP.

This hypothesis was checked by removing N-AFP antibodies from the specific L-AFP antibodies with a preliminary migration of N-AFP included in the intermediate gel for 8 h before placing N-AFP or L-AFP in the well (Fig. 7C). This treatment resulted in the production of a rocket with only the L-AFP from (wells 2 and 3), while the N-AFP form was no longer seen (wells 0 and 1).

Such results and changes in AFP immunoreactivity can lead to apparent differences in AFP quantitation as a function of the free fatty acid environment. This variation depends largely on the biological fluid or the pathophysiological situation analyzed (Table I). Previous studies, using Laurell immunoelectrophoresis and anti-rodent or anti-human polyclonal antibodies, showed that the quantitation of AFP incubated with polyunsaturated fatty acid was reduced or difficult to determine because of the fuzzy appearance of the rockets. Similar results were obtained even when the AFP-fatty acid mixture had been treated with charcoal-Dextran suspension to remove unbound lipid.

Purified human AFP was quantified by RIA (Abbot and CEA) and ELISA using monoclonal antibody in the presence of polyunsaturated fatty acid (Fig. 8). There was a progressive loss of immunoreactivity with increasing polyunsaturated fatty acid (22:6) concentration. The apparent concentration of purified AFP dropped sharply up to a concentration of 0.2 μmol 22:6 per ml in the incubation mixture. Higher FA concentrations produced no further significant modification of AFP immunoreactivity (Fig. 8A). Human AFP in different biological fluids (cord serum, hepatoma serum and fetal serum) was assayed in the presence and absence of increasing concentrations of exogenous polyunsaturated fatty acid (22:6) (Fig. 8B).

The estimations of AFPs in fetal and hepatoma serum were FA dose-dependent. The estimated AFP concentration in the presence of 3 μmol FA per ml was only 10% (fetal serum) or 50% (hepatoma serum) of the control value measured in the absence of exogenous FA. The estimation of AFP in cord blood was the least affected by exogenous FA (95% control at all the FA concentrations tested). These results may be correlated with the level of fatty acid in each biological fluid (Table I). The lowest level of fatty acid was found in cord serum, which also had the lowest relative percentage of polyunsaturated fatty acid, while the fetal serum had the highest fatty acid content and the highest relative percentage of polyunsaturated fatty acid.

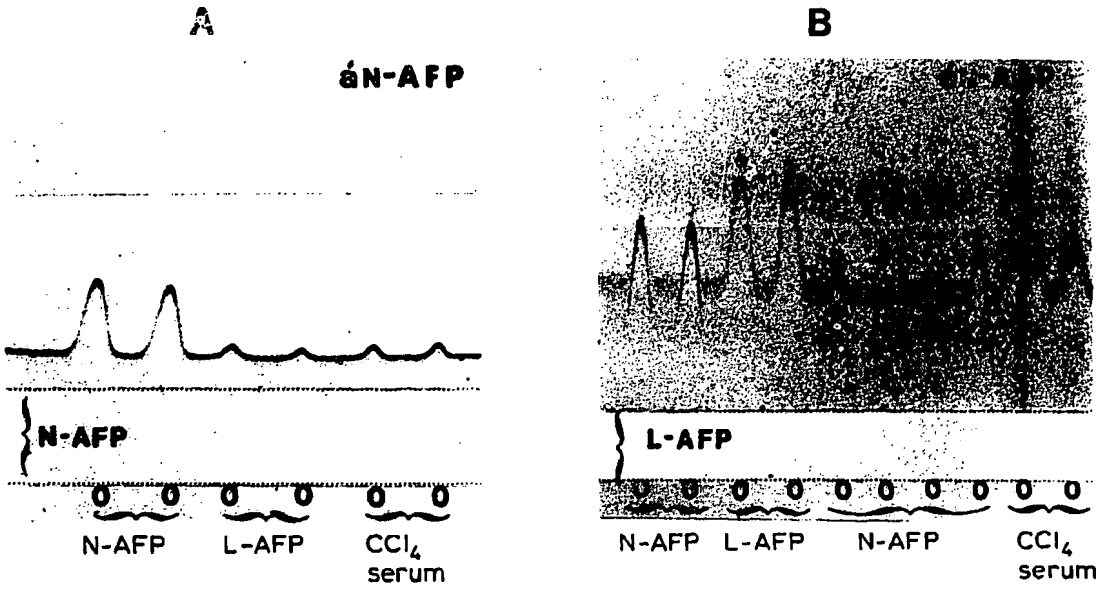


Fig. 6. Rocket-line immunoelectrophoresis of rat N-AFP (A) and L-AFP (B) against anti-rat N-AFP antibodies (àN-AFP). (A) N-AFP (500 ng) was included in the intermediate gel strip. The sample wells contained: N-AFP (50 ng), L-AFP (50 ng), 5 μl of CCl_4-treated-rat serum. The L-AFP was prepared by preincubating AFP with 22:6 (5 μmol 22:6/nmol AFP). 1% anti-rat N-AFP antiserum (àN-AFP). (B) L-AFP (2.5 μg) was included in the intermediate gel strip. The sample wells contained: N-AFP (100 ng), L-AFP (100 ng), N-AFP used as standards (37, 25, 12 and 50 ng), 5 μl of CCl_4-treated rat serum. Antiserum: anti-N-AFP antibodies (àN-AFP) (1%).

Discussion

AFP has been described as a flexible monomeric three-domain structure with hydrophilic external surfaces and large, deep hydrophobic pockets [33]. The present binding, spectral and immunochemical studies show that the high-affinity ligands, unsaturated fatty acids, can induce conformational changes in AFP.

Previous studies have shown that the binding of estrogen to rodent AFP is not affected by saturated fatty acid but is inhibited by unsaturated fatty acid in a dose-dependent manner and as a function of their degree of unsaturation [5]. The present binding study shows that the parameters, K_a and n, for E2 binding to rat AFP are modified in the presence of increasing concentrations of unsaturated fatty acid. The K_a varied non-linearly as a function of increasing unsaturated fatty acid concentration and this relationship may be a reflection of 22:6-induced changes in AFP conformation. This results might also indicate that there are

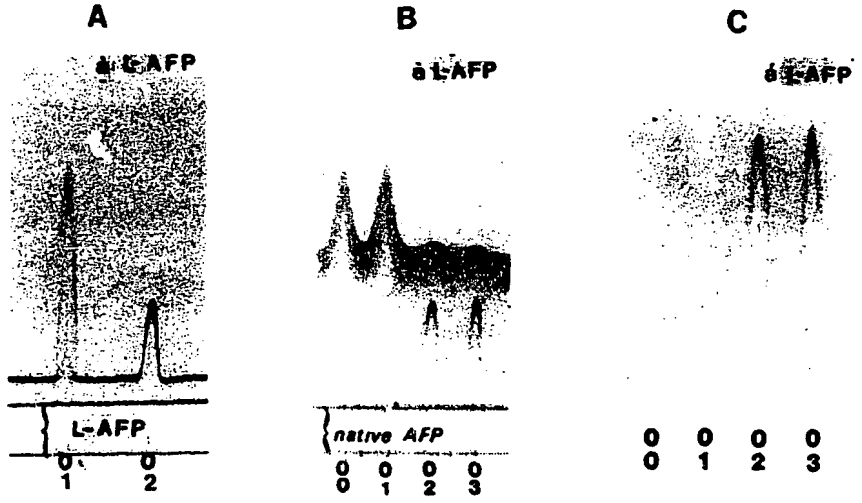


Fig. 7. Immunoreactivity of rat N-AFP and L-AFP against anti-rat L-AFP antibodies. Rocket-line immunoelectrophoresis studies. (A) L-AFP (10 μg) was included in the intermediate gel. The L-AFP was prepared by preincubating AFP overnight with 22:6. The sample wells contained: N-AFP (250 ng) (well 1), L-AFP (250 ng) (well 2). (B) N-AFP (10 μg) was included in the intermediate gel. The sample wells contained N-AFP (250 ng) (wells 0 and 1) L-AFP (250 ng) (wells 2 and 3). (C) N-AFP antibodies were removed from the specific L-AFP antibodies by a preliminary migration of N-AFP (20 μg) included in the intermediate gel for 8 h. This latter was subsequently removed before planing N-AFP (500 ng) (wells 0 and 1) or L-AFP (500 ng) (wells 1 and 2) in the wells.

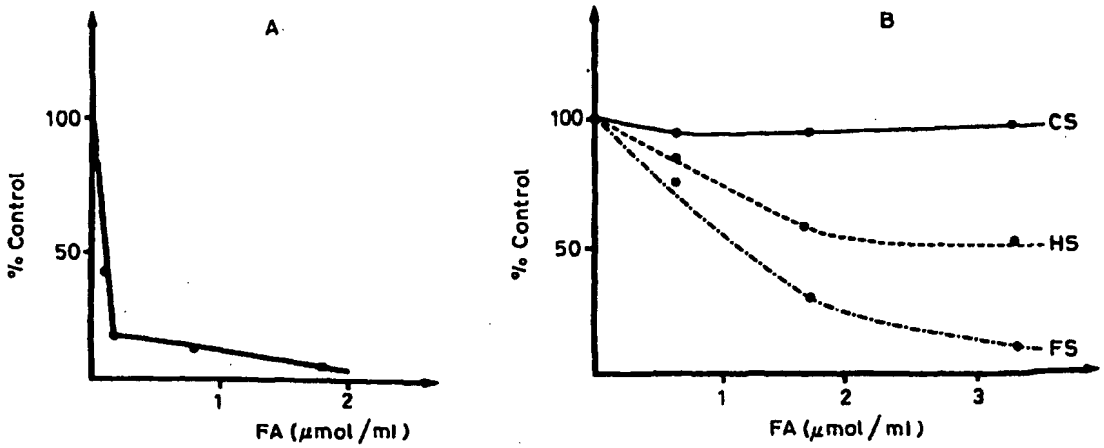


Fig. 8. (A) Radioimmunoassay of AFP purified from human fetal serum, in the presence of increasing concentrations of 22:6. RIA was performed with the Abbot kit. Results are expressed as percent of the control (100%) measured without 22:6. The concentration of AFP control was 0.11 nmol/ml. (B) Radioimmunoassay of human AFP in cord serum (CS), hepatoma serum (HS) and fetal serum (FS) in the presence of increasing concentrations of exogenous 22:6. RIA was performed with the Abbot Kit. The reaction mixtures contained the appropriate concentrations of unsaturated FA plus an aliquot of serum equivalent to approx. 10 μg AFP. The results were expressed as % of their respective controls. The concentration of AFP in different sera before incubation with 22:6 was taken as 100%.

several fatty acid binding sites on rodent AFP ($n = 4$ for 20:4, data not shown) with two classes of affinity binding site [7]. Thus, about 7.6 nM 22:6 would saturate the sites that do not affect the K_d of E2 binding (non-competitive binding), while over a range of 38–760 nM 22:6, the K_d of E2 varied linearly, suggesting that as with tryptophane methyl ester [34], 22:6 induces competitive inhibition. The K_d for the E2-AFP complex changed linearly with increasing amounts of either E1 or E2, suggesting that, there is a reciprocal competition between the steroid hormones. Thus unsaturated fatty acids appear to affect the conformation of AFP, specifically influencing its estrogen-binding activity. Similar effects of unsaturated fatty acid have been reported for corticosteroid-binding globulin (CBG) and anti-estrogen sites [12,35].

Analysis of multi-wavelength HPLC output profiles showed that the absorption minima of rodent and human L-AFPs incubated with unsaturated fatty acid were red-shifted and broadened in a dose-dependent manner (5 nm to 10 nm) and that the absorbance minimum was greater for L-AFP than for N-AFP. The changes in the multiwavelength ratio plots for rat and human AFP with fatty acid concentration seem to be very similar. They were not changed by incubation with saturated fatty acid. These variations in the UV spectral properties of rodent and human AFP with the unsaturated fatty acid environment may reflect the absorption of the unsaturated fatty acid bound to AFP. They may also provide indices of unsaturated fatty acid-induced conformational changes in AFP. Similarly, human AFP conformational states with characteristic changes of the accessibility of the hydrophobic sites has been demonstrated as a function of acid or alkaline environments [33].

The immunological results may also provide additional evidence for FA-mediated conformational changes of AFP. High-titer-specific polyclonal antibodies against N-AFP and L-AFP preparations were used to check the hypothesis that there are several conformational states corresponding to different forms of lipidated AFP (holoforms).

N-AFP antibodies recognized fewer epitopes on L-AFP than on N-AFP (Fig. 6), indicating that either FA directly masks some epitopes of N-AFP, giving rise to a partial identity between N-AFP and L-AFP, or that FA binding is associated with a conformational change in N-AFP to cause rearrangement of surface antigen sites and bring about quantitative and qualitative alteration in the reaction with antibody. The addition of FA to N-AFP without prior incubation did not disturb the immunodetection of N-AFP, and charcoal-dextran treatment to remove the unbound FA did not change the L-AFP holoform recognition by anti-N-AFP antibodies. Thus, the perturbation of the immunoprecipitation process was not due to the FA alone; it is more likely that this is caused by a conformational change of AFP in the presence of FA. L-AFP antibodies reacted poorly with N-AFP in the rocket-line system, while reacting well with the L-AFP holoform.

The N-AFP and L-AFP injected as immunogens probably correspond to equilibrium mixtures of different, more or less lipidated, states of AFP. Such equilibrium states could explain why the L-AFP was slightly recognized by anti-N-AFP antibodies and, why N-AFP was slightly recognized by anti-L-AFP. Line immunoelectrophoresis and the use of specific polyclonal antibodies offer an excellent screening procedure for detecting, characterizing and isolating the class of antibodies directed against each specific conformational state of a

protein. Thus, the absorption of the polyclonal L-AFP antibodies in situ by N-AFP antigen in the gel strip shows that it is possible to isolate classes of antibodies which react specifically with the L-AFP form (Fig. 7C). These results provide an additional argument for the being conformational transition states of AFP which are dependent on its hydrophobic environment.

The biological environment of the AFP, especially the NEFAs which varies greatly during ontogenesis [36], oncogenesis and in pathological situations, could confer particular conformational states on this oncofetal protein. This is emphasized by the immunodetection of AFP in different biological fluids. The immunopattern of AFP from CCl_4-treated rat serum was close to that of L-AFP holoforms, suggesting that the conformation of this pathological AFP is different from that of fetal AFP. The AFP immunopattern in CCl_4-treated rats may be correlated with the fact that the serum of these rats had a relatively high percentage of polyunsaturated FA (44%) and low AFP concentrations (μg/ml) compared to fetal serum (23% polyunsaturated FA, mg/ml AFP).

The apparent values of AFP obtained by radioimmuno-assays of various biological fluids were modified differentially by exogenous unsaturated FA. This could be explained by the existence of different conformational states of the protein more or less sensitive to the effect of exogenous FA. The difference in sensitivity to FA of the various AFP could be related to their NEFA environment in each biological fluid studied. Indeed, the free fatty level in cord blood serum is much lower (10-fold) than that of hepatoma or fetal serum. Moreover, the relative percentage of polyunsaturated fatty acid was very low in cord blood serum (5%) comparatively to hepatoma serum (8%) and fetal serum (23%). However, these results may also be due to other factors, such as the presence of different isoforms of AFP in each of the sera, with the combination of forms found in the cord blood serum being much less sensitive to fatty acids. The original choice of AFP cord serum as the international standard could be explained by such results [37]. Some of the problems encountered in the measurement of AFP may, in some pathophysiological situation, be linked to these differences in FA sensitivity [38].

These FA-induced changes in the spectral, electrophoretic and immunological behaviour of AFP and in its functional properties (binding) tend to indicate that this oncofetal protein has an enormous adaptative and interactive capacity towards the environmental changes which occur during normal and pathological development.

Hence endogenous or exogenous (nutritional) NEFAs may modify the three-dimensional structure of AFP, with the reciprocal interaction of saturated and unsaturated fatty acids in the immediate AFP environment contributing to the formation of transient forms of AFP (holoforms). Some of these holoforms could interact with specific membrane receptors and become internalized [21,39]. The presence of multiple holoforms may thus explain the inhibitory or stimulatory effect of AFP on the response of immune cells [14–23] or estrogen target cells [20,40]. These transient forms could comprise one of the keys to the multiple functional potentiality of the AFP.

Acknowledgements

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Exhibit C

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In vivo transient rise in plasma free fatty acids alters the functional properties of α-fetoprotein

M. Haourigui, N. Thobie, M.E. Martin, C. Benassayag and E.A. Nunez

U.224, INSERM, Faculté de Médecine Xavier Bichat, Laboratoire de Biochimie, Paris (France)

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Key words: Unsaturated fatty acid; Heparin; α-Fetoprotein; Protein conformation; Estrogen binding; Immunodetection; (Human)

Previous in vitro studies have shown that unsaturated fatty acids (UFA) induce conformational changes in rodent and human α-fetoprotein (AFP). To determine whether such changes in the binding and immunological properties of rat AFP also occur in vivo, plasma free fatty acid (FFA) concentrations were increased in young male rats (15, 21 and 28 days old) by acute i.v. injection of heparin (200 IU/kg). Plasma estrogens (estrone and estradiol) did not change after injection of heparin. There was a large increase in plasma FFA 10–20 min post-heparin injection, with a return to normal 60 min later. This transient rise in FFA plasma was associated with a 50% drop ($P < 0.001$) in the binding of estradiol to rat AFP of 15-, 21- and 28-day-old rats by reducing the number of binding sites ($P < 0.001$), leaving the affinity constant (K_a) unchanged. FFA extracts from post-heparin plasma induced similar changes in estradiol binding to purified rat AFP. The rise in plasma FFA induced a loss of AFP immunoreactivity, in 21- ($P < 0.001$) and 28-day-old rats ($P < 0.001$), but not in 15-day-old rats. This age-dependent response correlated with the FFA/AFP molar ratio (38 in 15-day-old rats, 388 in 21-day-old rats, and 5600 in 28-day-old rats). These results indicate that an in vivo rise in FFA induces rapid and reversible conformational changes in AFP which may modulate the endocrine and immune function of this oncofetal protein.

Introduction

There is now considerable evidence that free fatty acids (FFA) play a major role in the transfer of hormonal information. They induce subtle specific changes in the binding of hormones to several plasma proteins, including murine α-fetoprotein (AFP) [1–8], human sex-binding protein (SBP) [9], human corticosterone-binding globulin (CBG) [10], and thyroxine-binding globulin (TBG) [11,12].

Previous studies have established that FFA inhibit estrogen binding to purified rat and mouse AFP. This inhibition is dose dependent, varies with their degree of unsaturation and results from a FFA-induced conformational change in AFP that is reflected in alterations in the immunological behaviour of this protein [7,13]. AFP from different species bind FFA, especially polyunsaturated fatty acids, with high affinity [4,5, 14,15], and some studies indicate that the functional properties of this protein depend on its endogenous FFA content, which in turn depends on its exogenous FFA environment [6,8]. These in vitro results led us to determine whether a single transient change in the plasma FFA concentration in vivo could affect the functional properties of the serum steroid binding protein, rat AFP.

To induce lipolysis in young male rats we used heparin, which is known to release hepatic lipase (HL) and lipoprotein lipase (LPL) from endothelium cells into the blood stream and to cause an increase in plasma free fatty acids [16].

In this study we have examined the influence of heparin-induced changes in plasma FFA on the physicochemical properties of AFP during two developmental periods of the rat, one in which the plasma concentration of AFP is high (15-day-old rats), and the second when the AFP concentration is lower (21-day-old and 28-day-old rats) [6].

The results indicate that the transient increase in plasma FFA induced by heparin causes significant but

Abbreviations: UFA, unsaturated fatty acids; AFP, α-fetoprotein; FA, fatty acids; FFA, free fatty acids; SBP, sex-binding protein; CBG, corticosterone-binding globulin; TBG, thyroxine-binding globulin; HL, hepatic lipase; LPL, lipoprotein lipase; RIA, radioimmunoassay.

Correspondence: E.A. Nunez, U.224, INSERM, Faculté de Médecine Xavier Bichat, Laboratoire de Biochimie, 16 rue Henri Huchard, 75018 Paris, France.

reversible conformational changes in the structure of AFP, which are reflected in altered binding properties and immunoreactivity.

Materials and Methods

Reagents

[6,7-^{3}H]Estradiol (55 Ci/mmol) and [2,4,6,7-^{3}H]estrone (98 Ci/mmol), purchased from the Amersham International (Amersham, UK) were 98–99% pure. They were regularly tested to ensure that level of purity.

Unlabelled steroids estradiol (E2) and estrone (E1) and standard saturated and unsaturated free fatty acids were purchased from Sigma (St. Louis, MO). Heparin (200 IU/ml) was from Choay, Paris, France.

Animals and plasma preparation

Male Wistar rats (Charles River 76419 Saint-Aubin-les-Elbeuf, France), 15 days old weighing 36 ± 5 g and 21 days old weighing 55 ± 11 g, were kept in a controlled environment (20–22°C, light period 08.00–16.00). The 21-day-old rats had free access to standard diet pellets and tap water and the mid-suckling rats (15 days) were kept with their mothers until the experiment.

Groups of 15 rats of each age were injected intravenously with heparin (200 IU/kg in 0.5 ml 0.9% NaCl) via the tail vein under light ether anaesthesia.

Control rats were given 0.5 ml 0.9% NaCl. All experiments were performed between 9 and 12 h. Rats were bled 10, 20 and 60 min after injection; the blood was collected in glass tubes containing trisodium citrate (1.26 ml) on ice and immediately centrifuged at 3000 × *g* for 20 min. The plasma was stored at −20°C until analysis.

Extraction of steroids and FFA

Unlabelled heptadecanoic acid, [^{3}H]E1 and [^{3}H]E2 tracers were added to plasma samples (0.5 ml) as internal standard, each sample was extracted three times with organic solvent (ethyl acetate/cyclohexane, v/v) and the aqueous phase was removed by freezing (−20°C). The organic extracts were pooled, evaporated to dryness, taken up in 1 ml of chromatography solvent (benzene/ethanol, 95:5, v/v) (solvent I) and applied to Sephadex LH20 microcolumn (0.5 × 6 cm) equilibrated with benzene/ethanol (95:5, v/v). Free fatty acids were first eluted with 2.9 ml of solvent I. Estrone (E1) was then eluted with 3.5 ml of solvent I followed by 1 ml of solvent II (benzene/ethanol, 90:10, v/v). The fractions were evaporated to dryness and dissolved in benzene (1 ml). The yields from the extraction and purification steps were about 80%, as measured by the radioactive E1 and E2 standards, and by the unlabeled 17:0 standard FFA.

FFA analysis

The FFA fraction was evaporated to dryness and methylated in boron trifluoride/methanol (Merck). The methylated fatty acids were chromatographed on a Packard chromatograph, model 439 (Chrompack, Holland-Packard), using a capillary column (WCOT fused silica CP-sil-8CB 25 m × 0.32 mm). The column temperature was 151°C for the first 5 min, increasing thereafter by 3°C/min to 239°C, and by 10°C/min to 290°C. The injector temperature was 260°C and the detector temperature was 280°C.

The response coefficients and concentrations of FFA were determined with a recording chromatography data processor (Chromatopac CR1AB, Packard model 604). The peak area ratios of each FFA from plasma extracts and *n*-heptadecanoic acid (17:0) were compared to the peak area ratios of each FFA standard and 17:0. The total FFA concentration was obtained by summing all methylated derivatives on chromatography.

Blanks from all the buffers and solvents were run in parallel; no interfering exogenous contaminant was observed.

Immunoassay (RIA) of estrone and estradiol

The Sephadex LH20-purified sample of E1 was quantified by radioimmunoassay using rabbit anti-E1 6-thyroglobulin serum (Miles, Yeda Ltd, Israel). Estradiol (E2) was assayed in plasma by the Delfia method (Pharmacia). At least two measurements were made for each serum sample. The detection limit for estrogens was 18 pM. All antibodies were 100% specific for the hormones measured.

The blanks obtained by running the solvents through the whole extraction procedure were tested by radioimmunoassay, they produce no interference with the specific antibody.

Radioactivity was determined on samples, dissolved in 4 ml PCSII (Amersham) and counted in an Packard 1500 liquid scintillation spectrometer with the internal standard for technique quenching evaluation.

Serological methods

Purified rat AFP and monospecific rat AFP antibodies were obtained as previously described [2,7]. AFP was quantified by the Laurell rocket electroimmunodiffusion technique [17]. The detection limit of AFP was 0.015 μM.

Crossed immunoelectrophoresis was performed by the method of Laurell as modified by Clark and Freeman [18].

Dimension 1. Plasma samples (5 μl) were electrophoresed in 1% agarose gels at 8 V/cm using Tris-barbital lactic acid electrode buffer (pH 8.6) at 14°C.

Dimension 2. It was run overnight, at right angles to the first separation with the same buffer at 14°C at 2

V/cm into 1% agarose containing 0.5% monospecific anti-rat AFP antibody.

The gels were dried and stained with Coomassie blue.

Binding studies

The binding of [^{3}H]estradiol to rat plasma proteins was measured by the batchwise gel equilibration technique of Pearlman and Crépy [19] in phosphate buffer (H_2KPO_4/HNa_2PO_4, M/15, pH 7.4 at 21°C). The combining affinity indices or 'C values' (l/g) measure the ability of a mixture of proteins to bind a small molecule and is given by the expression: $C = (B/U)(1/P)$ where B, U and P are the concentrations of bound ligand (nM), unbound ligand (nM) and proteins (g/l), respectively [20].

Scatchard plots [21] were used to determine the affinity constant (K_a) and the apparent concentration of binding sites (nM). A series of assays with fixed amounts of plasma (2 μl for 15-day-old rats, 5 μl for 21-day-old rats) or purified rat AFP (3 μg = 29 nM) and [^{3}H]E2 (100000 cpm = 1.8 nM), plus increasing quantities of unlabelled E2 (0.9 to 184 nM) were performed.

Competition studies on the plasma free fatty acids fraction from controls or heparin-treated rats were carried out with fixed amounts of pure rat AFP (29 nM) and [^{3}H]E2 (100000 cpm = 1.8 nM), plus increasing quantities of organic solvent FFA extract from 21-day-old rats plasma at 10 min (♯ 1.5 mM) and 60 min (♯ 0.7 mM) post-injection.

Protein determination

Protein concentrations were assayed as described by Lowry et al. [22], with rat serum albumin as standard.

Statistical analysis

All data are reported as means ± S.E. Student's t-test was used to compare means. Results were considered significant when the probabilities were: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$.

Results

Plasma free fatty acid concentrations

The plasma free fatty acid concentrations of control 15- and 21-day-old rats were similar at all times after saline injection (Fig. 1), and were not significantly different from those of uninjected rats (data not shown).

The plasma FFA concentration of mid-suckling rats (15 days) increased significantly 10 min ($P < 0.001$), 20 min ($P < 0.01$) and 60 min ($P < 0.05$) after heparin injection.

The plasma FFA concentration of weaning rats (21 days) increased significantly only 10 min ($P < 0.001$) and 20 min ($P < 0.01$) after heparin injection. The increases occurred equally in all FFA classes, saturated and unsaturated (data not shown).

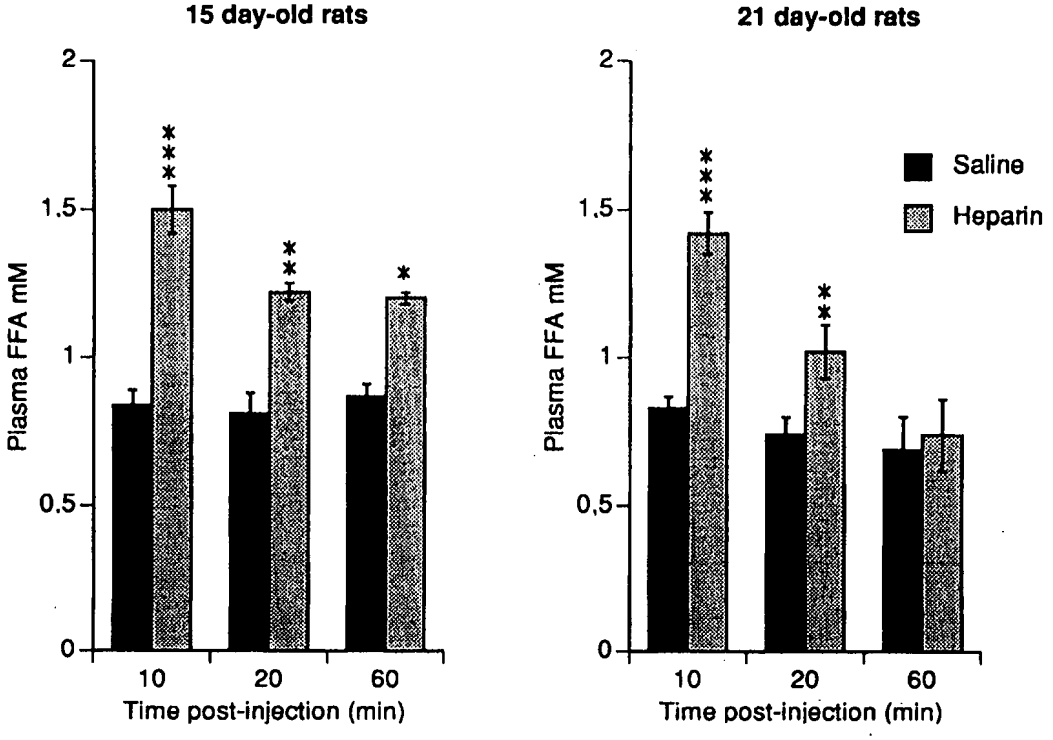


Fig. 1. Free fatty acid concentrations in the plasma of saline-injected control (■) and heparin-treated (□) rats. Values are means ± S.E. for 15 rats. Control and treated rats were compared using Student's t-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

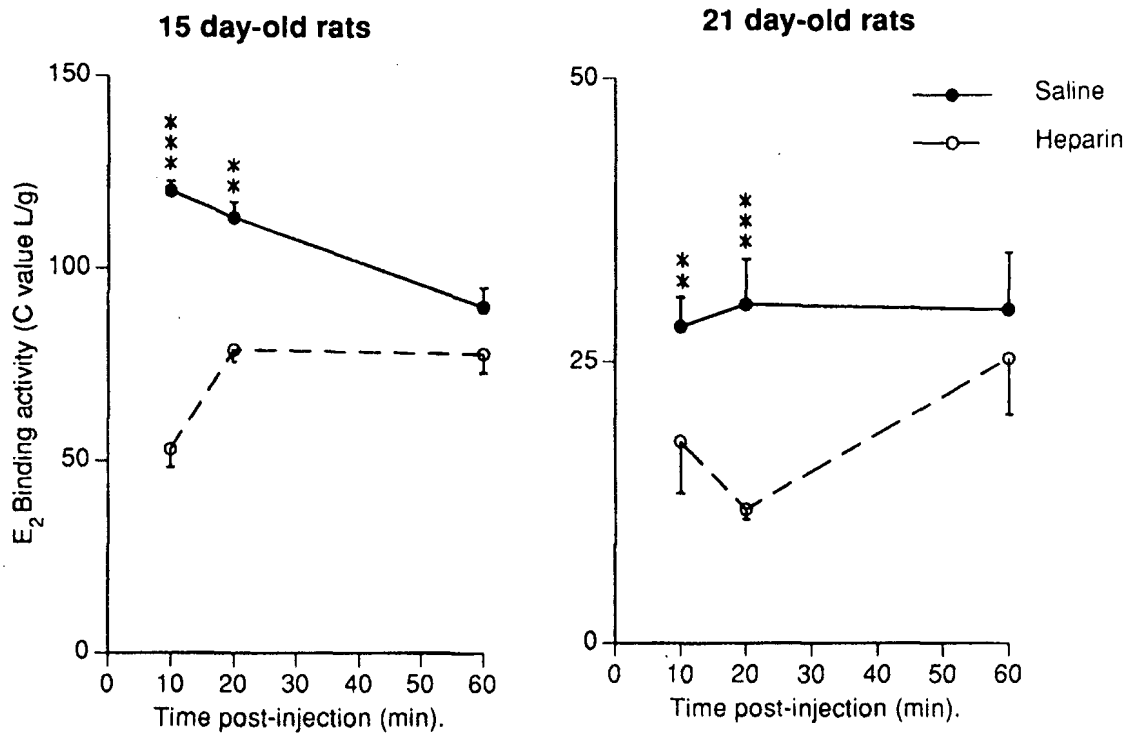


Fig. 2. Binding activities of plasma AFP (C values, l/g) following injection of 15- and 21-day-old rats with saline (•) or heparin (○). Estradiol binding was determined at 21°C by batch-wise gel equilibrium dialysis. The incubation mixture contained 2 μl (15-day-old rats = 42 mg protein/ml) or 5 μl (21-day-old rats = 55 mg protein/ml) plasma and [^{3}H]E2 (100000 cpm) in H_2KPO_4/Na_2HPO_4 buffer (M/15, pH 7.4). Values are means ± S.E. for 15 rats. Control and treated rats were compared using Student's t-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. We have checked that the addition of heparin (50–200 IU/mg of plasma protein) did not influence in vitro the E2 binding to AFP in the plasma of 15- and 21-day old rats.

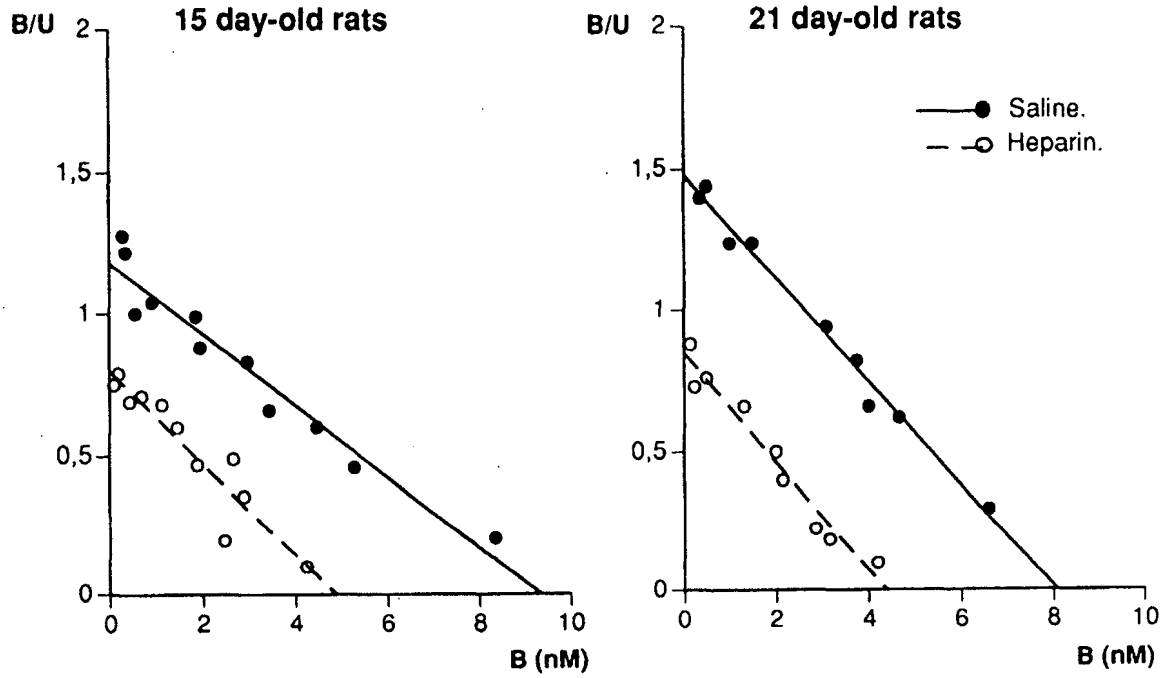


Fig. 3. Scatchard plots with Rosenthal correction for the interaction of [^{3}H]E2 binding with plasma AFP from control (•) and heparin-treated (○) 15- and 21-day-old rats. (A) 15-day-old rats. 2 μl of control or heparin-treated plasma taken 10 min post-injection was incubated with [^{3}H]E2 (100000 cpm) and 0.9–184 nM of E2, in H_2KPO_4/HNa_2PO_4 buffer (M/15, pH 7.4). (B) 21-day-old rats. 5 μl control or heparin-treated plasma taken 20 min post-injection was incubated as in A. B, bound steroid; U, unbound steroid. The E2 binding parameters K_a and nM are shown in Table I.

Plasma concentration of estrogens in heparin-treated rats

The plasma E2 and E1 concentrations of control rats were three times higher in the 15-day-old rats (3.24 ± 0.2 nM and 4.2 ± 0.19 nM) than in the 21-day-old rats (1.7 ± 0.2 nM and 1.19 ± 0.10 nM, respectively). Heparin treatment did not significantly alter the plasma E2 and E1 concentrations of either the 15-day-old (E2, 3.5 ± 0.04 nM; E1, 4.45 ± 0.13 nM) or the 21-day-old rats (E2, 1.72 ± 0.02 nM; E1, 1.13 ± 0.1 nM) at any time post-heparin injection.

Estradiol (E_2) binding to AFP

The E2 binding to control and heparin-treated rat plasma, expressed as C values (l/g), is shown in Fig 2. The C values for [^{3}H]E2 binding to AFP in post-heparin plasma from 15- and 21-day-old rats were both significantly lower 10 min ($P < 0.001$ and $P < 0.01$) and 20 min ($P < 0.01$ and $P < 0.001$) after heparin injection than those of control plasma. But the C values of E2 had returned to control values in heparin-injected rats by 60 min post-injection. These results indicate that the heparin-induced decrease in E2 binding to AFP is reversible and parallels the transient change in plasma FFA.

The association constant (K_a) and number of binding sites (nM) for E2 binding were determined by Scatchard analysis. Fig. 3 shows the Scatchard plot of control and post-heparin plasma from 15- and 21-day-old rats at 10 min and 20 min respectively, and Table I summarizes the E2 binding parameters at different times.

The decrease in C values for [^{3}H]E2 binding to AFP caused by heparin-induced lipolysis seems to be mainly due to a significant decrease in the number of binding sites ($P < 0.001$), while the affinity constant (K_a) was not affected.

The number of E2 binding sites (nM) in control and heparin-treated rats were the same at 60 min post-injection, in agreement with the observation that the C

TABLE I

Association constant (K_a) and number of binding sites (nM) from Scatchard analysis of [^{3}H]E2 binding to control and heparin-treated plasma from 15- and 21-day-old rats

		K_a (10^8 M^{-1})	nM
15-day-old rats			
control	10 min	1.3 ± 0.1	8.35 ± 0.35
	60 min	1.3 ± 0.2	8.00 ± 0.45
heparin	10 min	1.3 ± 0.2	5.22 ± 0.03 ***
	60 min	1.6 ± 0.4	6.65 ± 0.05 **
21-day-old rats			
control	10 min	1.5 ± 0.3	8.50 ± 0.20
	20 min	1.4 ± 0.4	8.35 ± 0.15
	60 min	1.5 ± 0.2	8.70 ± 0.50
heparin	10 min	1.6 ± 0.2	5.35 ± 0.15 **
	20 min	1.5 ± 0.3	4.38 ± 0.12 ***
	60 min	1.5 ± 0.2	7.75 ± 0.25

** $P < 0.01$.
*** $P < 0.001$.

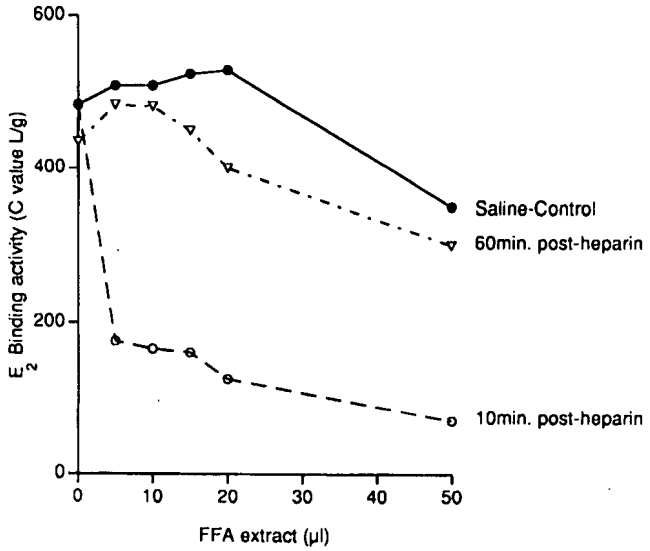


Fig. 4. Effect of increasing FFA extracted from saline or heparin-treated plasma on E2 binding activities of pure rat AFP. 3 µg (29 nM) rat AFP was incubated with [^{3}H]E2 (100000 cpm) in the presence of FFA extracts of plasma from 21-day-old rats, taken 10 min after saline (0.75 µmol/ml) (●), or heparin post-injection (1.47 µmol/ml) (○) or FFA extracts of plasma taken 60 min after heparin injection (0.65 µmol/ml) (△).

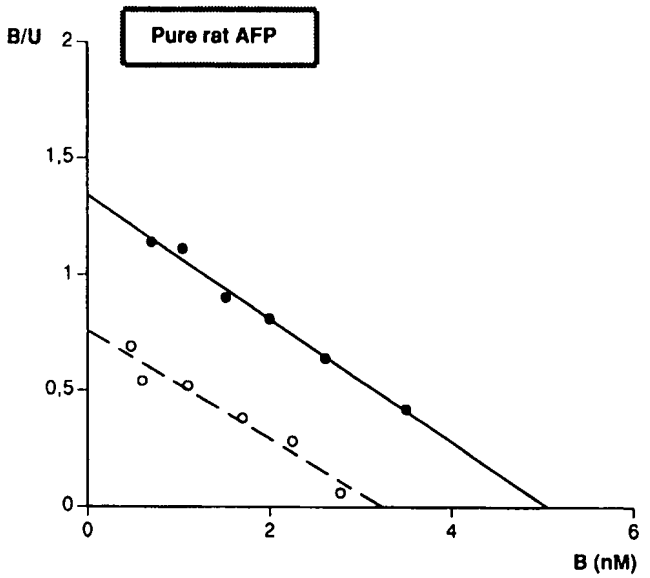


Fig. 5. Scatchard analysis of [^{3}H]E2 binding to purified rat AFP with (○) or without (●) 5 µl (7 nmol) FFA extracted from the plasma of 21-day-old rats taken 10 min after heparin injection. 3 µg (29 nM) purified rat AFP was incubated for 1 h at 21°C with [^{3}H]E2 (100000 cpm) and E2 (0.9–184 nM). Each point is the mean of duplicate assays. B, bound estradiol; U, unbound estradiol. Without 5 µl FFA extract, $K_a = 2.60 \cdot 10^8$ M^{-1} and nM = 5. With 5 µl FFA extract, $K_a = 2.34 \cdot 10^8$ M^{-1} and nM = 3.

values both heparin-treated and control 21-day-old rats were not statistically different at this time. Thus, the decrease in E2 binding sites observed at 10 min and 20 min was reversible at 60 min and correlated with the FFA level.

The E2 binding (C values) to AFP in the plasma of 15- and 21-day-old control rats was not disturbed in vitro by the addition of heparin (50 IU to 200 IU/mg of plasma protein) (see legend of Fig. 2).

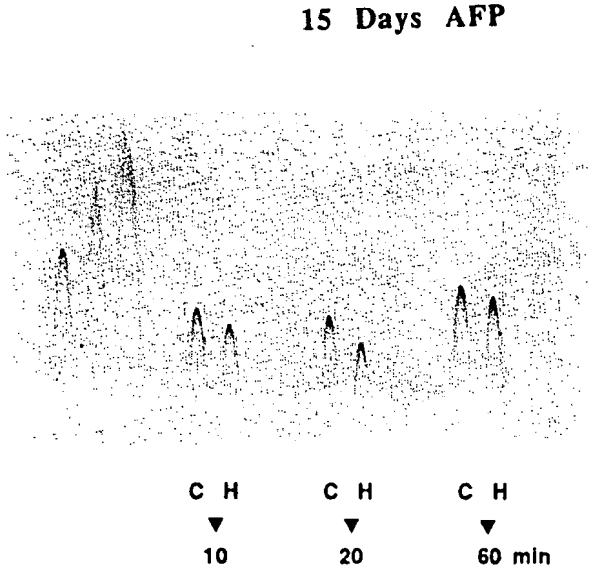


Fig. 6. Immunoquantification of AFP from 15- and 21-day-old rat plasma, taken 10, 20 and 60 min after saline or heparin injection. 5 μl control (C) or heparin-treated plasma (H) were electrophoresed overnight in 1% agarose containing 0.5% anti-rat AFP antibody at 8 V/cm using Tris-barbital lactic acid electrode buffer (pH 8.6) at 14°C. The first four wells contained pure rat AFP (10, 13.3, 20 and 26.6 μg/ml, respectively).

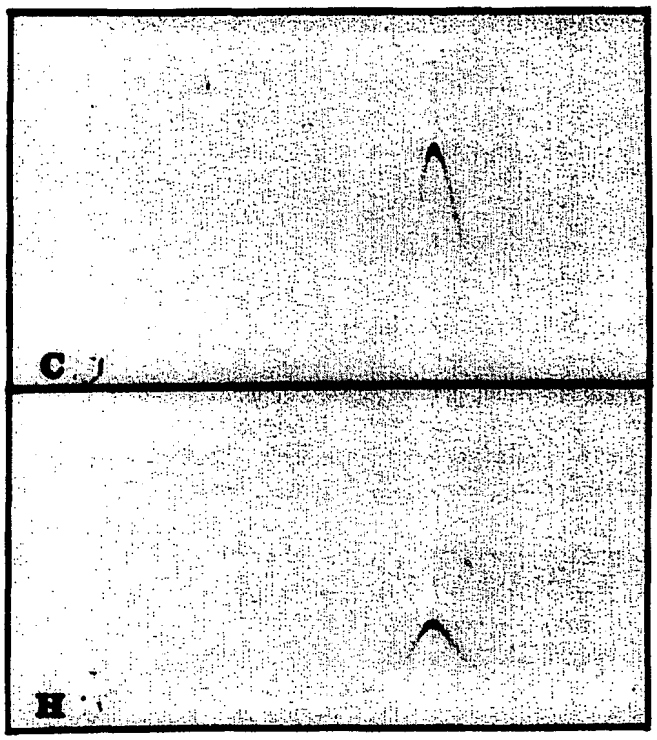


Fig. 7. Crossed immunoelectrophoresis of AFP in control (C) and heparin-treated plasma (H), taken 20 min after heparin injection. First dimension gel contained 5 μl control (C) or heparin-treated plasma (H). Second dimension gel contained 0.5% anti-rat AFP antibody. The gels were dried and stained with Coomassie blue.

Effect of FFA extracts of post-heparin plasma on estradiol binding to purified rat AFP in vitro

FFA extracts (1.47 μmol/ml) of plasma taken from 21-day-old rats 10 min after heparin injection produced a dose-dependent inhibition of E2 binding to rat AFP (C values, l/g) (Fig. 4). The inhibitory potency (IC_{50} values) for E2 binding was 20-fold higher for FFA extracts of plasma taken 10 min than those taken 60 min after heparin injection.

Scatchard analysis of E2 binding to pure rat AFP in the presence of 5 μl FFA extracts (7 nmol) of plasma from 21-day-old rats taken 10 min after heparin treatment (Fig. 5) indicated that the FFA extracts inhibited E2 binding to rat AFP, as in vivo with a decrease in the concentration of E2 binding sites (nM) and no significant change in K_a.

Immunoquantification of AFP

The concentrations of AFP in the plasma of control and heparin-treated rats were assessed by rocket immunoelectrophoresis using monospecific anti-AFP antibodies. Lipolysis appeared to have no statistically significant effect on the immunological properties of AFP from mid-suckling 15-day-old rats (Fig. 6 and Table II). By contrast there was a significant decrease

TABLE II

	FFA (μM)	Apparent AFP concentration (μM)	'C values' (l/g)	Apparent FFA/AFP molar ratio
Control rats				
15 days	840 ± 50	22 ± 1.28	108.0 ± 9.0	38
21 days	750 ± 70	1.93 ± 0.07	30.0 ± 4.0	388
28 days	340 ± 50	0.06 ± 0.01	0.9 ± 0.1	5600
20 min heparined rats				
15 days	1500 ± 80	22 ± 2.14	53.0 ± 4.0	68
21 days	1020 ± 90	0.77 ± 0.07	12.0 ± 0.7	1325
28 days	640 ± 40	0.014 ± 0.01	0.5 ± 0.1	45700

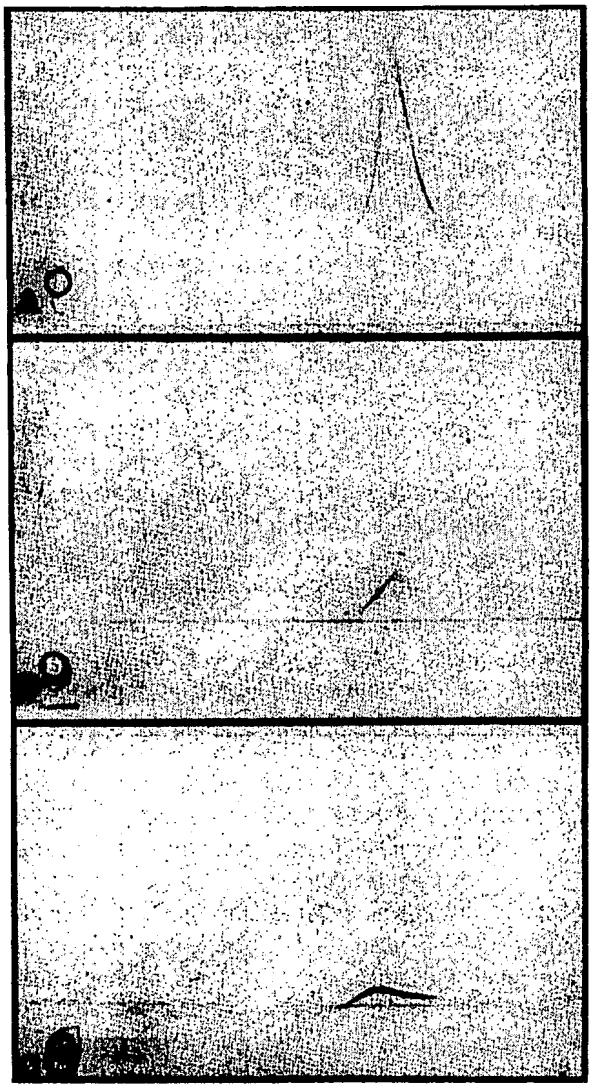


Fig. 8. Effect of FFA on purified AFP immunoreactivity in vitro. Crossed immunoelectrophoresis was carried out on AFP alone (A), AFP incubated with FFA extracted from heparin-treated rat plasma (B) and AFP incubated with a mixture of FFA standards (C). Antiserum: rabbit anti-rat AFP (0.3%). (A) Purified rat AFP (1.8 pmol). (B) Purified rat AFP (1.8 pmol) incubated overnight at 4°C with 104 nmol FFA extracted from the plasma of 21-day-old rats 10 min after heparin injection (FFA extract composition: 33% saturated FA, 14% monounsaturated FA, 53% polyunsaturated FA). (C) Purified rat AFP (1.8 pmol) incubated overnight at 4°C with 50 nmol standard FFA mixture (33% saturated FA, 14% monounsaturated FA, 53% polyunsaturated FA).

($P < 0.001$) in the amount of AFP in the plasma of 21-day-old rats (Fig. 6), especially 20 min after heparin injection (2 μM in control plasma and 0.77 μM in heparin-treated plasma). The amounts of AFP in the plasma of control and heparin-treated rats were essentially the same 60 min post-injection (Fig. 6) (2 μM in control and heparin-treated rats).

Fig. 7 shows the crossed immunoelectrophoresis patterns for AFP in the plasma from 21-day-old rats taken 20 min after saline or heparin injection. The immunorecognition of AFP by anti-AFP antibodies is clearly decreased after heparin-induced lipolysis, without any significant change in the mobility of AFP. Similar results were obtained with plasma AFP from 28-day-old rats, especially 10 min and 20 min after heparin injection (data not shown).

The immunoreactivity of AFP was unaffected by the circulating concentration of heparin.

The immunological behaviour of purified AFP loaded with FFA extracted from heparin-treated plasma from 21-day-old rats or FFA standard mixture (with a composition close to that of the extract) was analysed to determine whether the decrease in immunoreactivity of heparin-treated plasma AFP was due to a FFA-induced conformational change in AFP.

Fig. 8 shows that both AFP loaded with FFA plasma extracts (Fig. 8b) and AFP loaded with a FFA standard mixture (Fig. 8c) were poorly recognized by anti-AFP antibodies directed against native AFP (i.e., AFP not loaded with FFA). Increasing the FFA standard mixture concentration also decrease the immunoreactivity of purified AFP in a dose-dependent manner (data not shown). Previous studies [7] have shown that adding FFA to purified AFP without prior incubation does not disturb the immunodetection of AFP.

Discussion

These results indicate that the transient rise in plasma FFA which occurs in vivo after heparin-induced lipolysis alters the functional properties of the steroid carrier protein, rat AFP. There was a clear relationship

between the rise in plasma FFA and conformational changes in AFP.

The significant increases (30 to 80%) in plasma FFA, especially 10 min and 20 min after heparin injection, without changes in plasma estrogen concentration, lead to a significant decrease (50%) in the estradiol-binding properties of rat AFP in 15-day, 21-day and 28-day-old rats. The E2-binding activities of heparin-treated rat AFP and saline-treated rat AFP were not significantly different 60 min after injection, which correlates well with transient nature of the rise in plasma FFA.

Thus, the inhibition of E2 binding to rat plasma AFP, which parallels the rise in plasma FFA concentration induced by heparin, is rapid and reversible.

The Scatchard analysis indicates that the apparent number of E2 binding sites on rat AFP were decreased by 50%, but the affinity constant (K_a) was unaffected in high-FFA plasma taken 10 and 20 min. post-heparin. The involvement of FFA in these changes was confirmed by the experiments showing that FFA extracts from the plasma of heparin-treated 21-day-old rats inhibited E2 binding to purified rat AFP in a dose-dependent manner. But the quantities required to produced 70% inhibition of E2 binding to purified rat AFP were 2-fold lower than with AFP in post-heparin plasma, suggesting that other proteins may interfere and/or bind FFA, as has been shown in vitro for human SBP, CBG, albumin, and lipoproteins [9,10,23–25].

FFA extracts markedly (70%) decrease E2 binding to pure rat AFP and mimic the in vivo situation, with Scatchard plots showing a 40% decrease in the number of E2 binding sites.

Previous in vitro studies using unsaturated FFA standards [7], suggest that the increased FFA saturates sites that do not affect the K_a of E2 binding sites (non-competitive binding).

The immunological results provide additional evidence for FFA-mediated conformational changes in AFP. The rise in FFA induces loss of immunoreactivity, especially in 21-day and 28-day-old rats. Polyclonal antibodies raised to native rat AFP (N-AFP) recognized fewer epitopes on heparin-induced lipolysis plasma AFP (L-AFP) than on N-AFP. Our studies performed with purified AFP show that FFA extracted from the plasma of heparin treated rats and FFA standard mixture both give rise to poor immunodetection of AFP.

Mixing heparin or FFA (120 nmol) with N-AFP without any incubation did not disturb the immunodetection of N-AFP. Thus, as previous in vitro studies [7], the perturbation of immunoprecipitation is probably caused by FFA binding to AFP, with a conformational change in L-AFP causing rearrangement of surface antigen sites to bring about quantitative and qualitative alteration in the reaction with antibody. However, the immuno-reactivity of the AFP in the plasma of heparin-treated 15-day-old rats was unchanged. The difference between immunoquantification of AFP in the post-heparin plasma from 15 day and older rats, and especially the misdetection of AFP in 21 day and 28 day plasma may be correlated with the fact that the plasma of 21- and 28-day-old rats contains a high concentration of FFA (0.75 mM and 0.35 mM, respectively) and a low AFP concentration (1.93 μM and 0.06 μM, respectively) (Table II). Thus, the immunological results indicate that the effect of FFA on AFP conformational changes depends on the FFA/AFP molar ratio: this ratio is 38 in 15-day-old rats, and 388 and 5600 in 21- and 28-day-old rats, respectively (Table II). However, other factors, such as the presence of different isoforms of AFP could be involved [6]. The combination of forms found in the plasma of 21- and 28-day-old rats may be more sensitive to fatty acids than that of 15-day-old rats.

Thus, the present in vivo data confirm the physiological relevance of our in vitro finding that unsaturated FFA induce conformational changes in rat AFP. As lipase activity is localized within cell membranes, it is likely that the concentration of fatty acids at these locations is higher than in the general circulation [26], and these in turn could influence AFP function. High FFA levels occur during stress, fasting, diabetes, obesity, and non-thyroidal illness [27–29]; under these circumstances the FFA/AFP ratio can be as much as 10-times that of normal. These findings also suggest that the FFA-induced changes in AFP hydrophobicity and conformation could modify AFP uptake by cell membranes such as lymphocytes [30], and so facilitate its internalization by target cells.

One of the most striking findings is the reversible effect of the rise of FFA on the binding and immunological properties of AFP, indicating that AFP could adapt to, and interact with the environmental changes which occur during ontogenesis, oncogenesis and in pathological situations. Our data also indicate that endogenous FFA from membranes may modify the 3-dimensional structure of AFP, and perhaps other serum hormone carrier proteins, such as SBP, CBG, or TBG, contributing to the formation of transient forms (holoforms). These changes could underly the multiple functional potentiality of AFP in endocrine, cell growth, and immune processes.

Acknowledgments

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Exhibit D

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The Presence of Fatty Acids in Human α-Fetoprotein*

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DAVID C. PARMELEE, MERLE A. EVENSON, AND HAROLD F. DEUTSCH

From the Departments of Physiological Chemistry and Medicine, University of Wisconsin Medical Center, Madison, Wisconsin 53706

α-Fetoprotein has been prepared from human fetal tissue by procedures utilizing DEAE-Sephadex, concanavalin A-Sepharose, and isoelectric focusing. A major and a minor component with isoelectric points of 4.7 and 5.3, respectively, have been isolated and are similar to those prepared under various conditions by other investigators. The 4.7 material contains 2.4 mol of fatty acids/mol of protein, whereas the minor component is fat-free. The relative amounts of fatty acid vary somewhat with different preparations. The ranges found in three isolates were as follows: palmitic acid (8 to 11%), stearic acid (2 to 5%), oleic acid (10 to 28%), linoleic acid (7 to 15%), arachidonic acid (12 to 39%), and 4,7,10,13,16,19-docosahexaenoic acid (16 to 42%). Human fetal serum albumin contained 0.7 mol of fatty acid/mol of protein, with arachidonic acid and the docosahexaenoic acid comprising only 11.4% of the total. Removal of fatty acids by treatment with charcoal converted α-fetoprotein into material with an isoelectric point of pH 5.3. Addition of arachidonic acid to the lipid-free protein restored it to protein with a pH 4.7 isoelectric point, typical of the major native component. The possible role of the fatty acids in α-fetoprotein on the inhibition of various lymphocyte functions is projected.

Human α-fetoprotein is a protein found in relatively high concentrations in the serum of fetal animals. It has attracted considerable attention due to its reappearance in several pathological conditions in adults. Abelev and co-workers (1) discovered that in many cases of hepatocellular carcinoma, the serum concentrations of α-fetoprotein were equal to fetal levels. This observation led to the classification of the protein as a tumor-specific embryonic antigen until more sensitive techniques demonstrated its increased serum concentration in tetratocarcinomas and some nonmalignant diseases (2–4). However, the protein remains an important clinical entity since the extremely high levels are unique to hepatocellular carcinoma.

The biological function of α-fetoprotein is not known, although various experimental observations have directed attention toward several possible roles. The specific binding of estradiol to the protein indicates that it may be important in the transport of this steroid (5) and it has been suggested to possibly play a role in protecting the embryo against the toxic effects of the high estrogen levels of the mother (6). More recently, a role of immunosuppression had been postulated by Murgita and Tomasi (7), who demonstrated that it suppresses both the primary and secondary *in vitro* antibody responses of lymphocytes. They also observed that α-fetoprotein can suppress allogenic and mitogen-induced lymphocyte transformation in mice (8). However, other workers have not found consistent effects of the protein on these immune responses and transformations (9, 10). The immunofluorescence studies of Dattwyler *et al.* (11) suggest that α-fetoprotein receptors are on the surface of certain T cell lymphocyte populations in mice. These findings, in conjunction with the abortogenic activity of antiserum to α-fetoprotein (12), indicate that it may be involved in preventing an immune response against fetal tissues or tumors. The similarities of α-fetoprotein and serum albumin as evidenced by sequence homologies (13) and immunological cross-reactivities (14) have suggested α-fetoprotein may serve as a fetal form of albumin by functioning to transport important biological molecules and to maintain osmotic equilibrium (15, 16).

The drastic conditions often employed in isolating α-fetoprotein may result in modification of its biological activity. For this reason, we have developed a relatively mild procedure for purifying α-fetoprotein from fetal tissue. The protein prepared by this method has been found to contain a variety of fatty acids, some of a type not noted in adult albumin or seen only in trace amounts. The fatty acids can be removed from the protein by using procedures analogous to those employed to remove fatty acids from albumin (17). This protein can be reconstituted with fatty acids to yield material with properties similar to the original preparation. The possible importance of the fatty acid components of α-fetoprotein in mediating the inhibition of lymphocyte functions is projected.

MATERIALS AND METHODS

Preparation of Con A[1]-Sepharose

Con A from 500 g of jack bean meal (Sigma) was purified on a column (3.5 × 90 cm) of Sephadex G-100 (Pharmacia) by the method of Agrawal and Goldstein (18). It was then coupled to 250 ml of Sepharose 4B (Pharmacia) by the cyanogen bromide procedure (19). A column of the resulting complex was equilibrated with pH 6.0, 0.1 M sodium acetate containing 1.0 M NaCl, 0.001 M $CaCl_2$, 0.001 M $MnCl_2$.

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[1] The abbreviations used are: Con A, concanavalin A; pI_e, isoelectric point; 16:0, palmitic acid; 18:0, stearic acid; 18.1, oleic acid; 18:2, linoleic acid; 20:4, arachidonic acid; 22:6, 4,7,10,13,16,19-docosahexaenoic acid; 24:1, nervonic acid.

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Purification of α-Fetoprotein

Preparation of Fetal Extract—The procedures described in the purification are based on 100 g of fetal tissue. Four- to five-month-old fetuses obtained immediately after therapeutic abortions served as starting material. Homogenates were prepared by blending fetal tissue with one-third their weight of 0.15 M NaCl in a Waring Blendor. The homogenate was clarified by centrifugation at 4400 × *g* for 5 min and the resulting residue was washed twice with the above weight of 0.15 M NaCl. The supernatants were combined to yield an extract of about 125 ml/100 g of starting tissue. The aggregated lipid material which formed during centrifugation was removed by filtration through glass wool. The fetal extract was then dialyzed against pH 6.2, 0.04 M potassium phosphate at 4°.

Chromatography on DEAE-Sephadex—The suspended material in the dialyzed extract was removed by centrifugation at 19,600 × *g* for 20 min. One liter of packed DEAE-Sephadex A-50 (Pharmacia) equilibrated with the dialysis buffer was then added to the resultant supernatant. After slow stirring at 4° for 6 h, the DEAE-Sephadex slurry was packed into a column (5.1 × 75 cm) and washed with 1 liter of the buffer. The adsorbed proteins, which included most of the α-fetoprotein, were eluted with the above phosphate buffer containing 0.5 M NaCl.

The protein fractions were pooled, dialyzed against the pH 6.2, 0.04 M potassium phosphate buffer, and concentrated to a volume of 10 ml in a Diaflo apparatus using a UM-10 membrane. The sample was then adsorbed on a column of DEAE-Sephadex equilibrated with the pH 6.2, 0.04 M potassium phosphate. Elution was effected by means of a linear gradient using 3 liters of pH 6.2, 0.04 M potassium phosphate buffer containing 0.15 M NaCl and an equal volume of this buffer containing 0.32 M NaCl. The presence of α-fetoprotein in the various fractions was determined by the use of a monospecific antibody[2] and fractions were pooled as indicated in Fig. 1.

Chromatography on Con A-Sepharose—α-Fetoprotein is difficult to separate from albumin due to similarity of molecular weight and isoelectric point. The absence of carbohydrate in serum albumin, however, permits removal of the α-fetoprotein by affinity chromatography on Con A-Sepharose (20). The fractions containing α-fetoprotein which eluted from the DEAE-Sephadex column (as shown in Fig. 1) were concentrated to a volume of 10 ml in the Diaflow apparatus and then dialyzed against pH 6.0, 0.1 M sodium acetate containing 1.0 M NaCl, 0.001 M $CaCl_2$, 0.001 M $MnCl_2$. The protein solution was then applied to a column of Con A-Sepharose equilibrated with this buffer, washed with approximately 0.7 column volumes of it, and then eluted with the same buffer containing 1% α-methylglucoside. The result shown in Fig. 2 was obtained. The first component which reacted only with the antibody to albumin eluted within 1 column volume after the sample application. Its elution was not dependent on the addition of the buffered 1% α-methylglucoside, whereas the second component eluted only after 1 column volume of the latter buffer. This component reacted only with specific antibody to α-fetoprotein.

Isoelectric Focusing—The fractions containing α-fetoprotein were pooled, exhaustively dialyzed against distilled water, and subjected to isoelectric focusing. As previously noted by other authors (16), two components, both reacting with antibody to α-fetoprotein were resolved (see Fig. 3). These fractions were pooled as indicated, dialyzed against distilled water, and stored at −20°.

Preparation of Antiserum—The major component of the highly purified preparation of α-fetoprotein was then used to prepare additional rabbit antiserum. The protein in complete Freund's adjuvant was administered to rabbits, each animal being given a total of 1 mg distributed equally in each of the hind leg foot pads as well as in two subcutaneous sites. Each week an additional 0.5 mg was given subcutaneously. After 4 weeks, the animals exhibited a serum antibody titer capable of detecting 10 μg of α-fetoprotein/ml by the interfacial ring test (21). The rabbits were then bled weekly by cardiac puncture and subcutaneous injections of 0.5 mg of α-fetoprotein in complete Freund's adjuvant were given concurrently to maintain the titer. Antiserum to serum albumin was prepared in a similar manner. These antibody preparations were employed to detect the presence of α-fetoprotein and albumin in various protein fractions and to ensure the purity of the α-fetoprotein preparations employed in various studies.

[2] This antiserum was a gift from Professor H. Hirai.

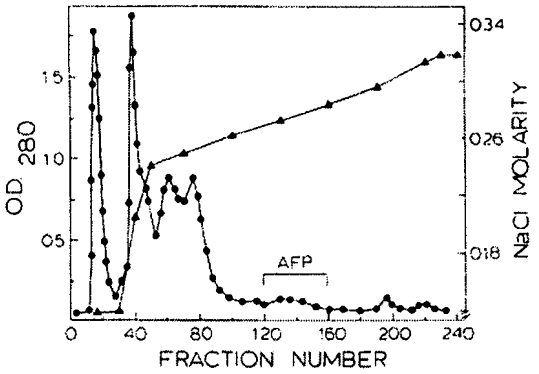


FIG. 1. Chromatogram illustrating the fractionation of a fetal extract from 475 g of tissue on a column (5.1 × 52 cm) of DEAE-Sephadex A-50 equilibrated with pH 6.2, 0.04 M potassium phosphate. The proteins were eluted at 4° by means of a linear gradient established by the addition of 3 liters of the buffer containing 0.32 M NaCl to 3 liters of one containing 0.14 M NaCl (▲——▲, molarity of added NaCl; ●——●, optical density at 280 nm). *AFP*, α-fetoprotein.

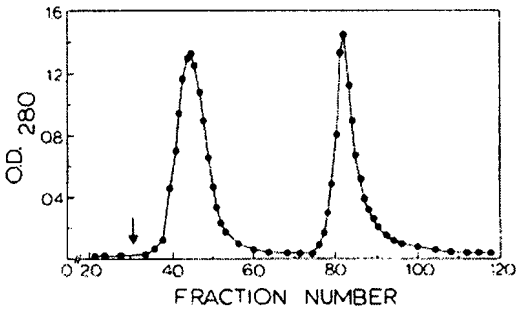


FIG. 2. Affinity chromatography on Con A-Sepharose of the α-fetoprotein fraction from 475 g of fetal tissue separated by DEAE-Sephadex. A column (2.5 × 41 cm) was equilibrated with pH 6.0, 0.1 M sodium acetate containing 1.0 M NaCl, 0.001 M $MnCl_2$, 0.001 M $CaCl_2$. The α-fetoprotein was eluted by the application of this acetate buffer containing 1% α-methylglucoside at the point indicated by the *arrow*. Fractions of 4 ml were collected using a flow rate of 6 ml/h/cm² (●——●, optical density at 280 nm).

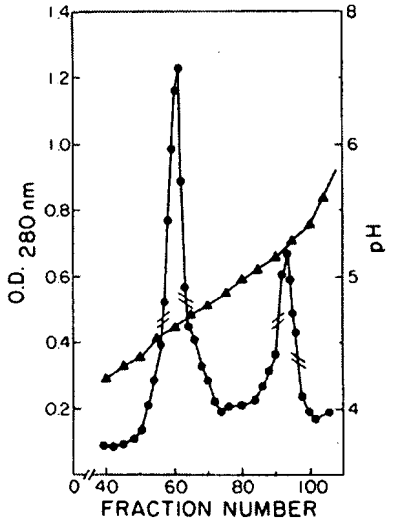


FIG. 3. Result for an isoelectric focusing experiment on the α-fetoprotein fraction (equivalent to 475 g of fetal tissue) separated by affinity chromatography on Con A-Sepharose. A pH 4 to 6 Ampholine was employed and fractions of 1 ml were collected (▲——▲, pH; ●——●, optical density at 280 nm). Fractions pooled to provide the pI_c 4.7 and 5.3 materials are indicated by the *hash marks* on the elution profiles.

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Quantitation of Fatty Acids

Extraction – Six milliliters of a solvent mixture consisting of 40 volumes of isopropyl alcohol, 10 volumes of *n*-heptane, and 1 volume of 1 N sulfuric acid (22) was added to 1.0 ml of α-fetoprotein solution containing from 0.5 to 3.5 mg of protein, to which 2 to 5 μg of *n*-heptadecanoic acid had been added as an internal standard. The mixture was mixed for 30 s in screw-capped test tubes (15 × 125 mm) to effect extraction of fatty acids. Two milliliters of distilled water was then added and the sample mixed for 15 s. Three milliliters of *n*-heptane was added and this solution again mixed for 15 s. A 3.5-ml aliquot of the upper organic phase was then removed, placed in the same type tube, and the solvent removed at 35° with a stream of nitrogen.

Methylation – The dried lipid extract was refluxed for 3 min with 1 ml of BF_3-MeOH (14% w/v)/BCl_3-MeOH (10% w/v) (Applied Science Labs., Inc.). One milliliter of *n*-heptane and 1 ml of distilled water were then added and mixed for 15 s. A 0.85-ml aliquot of the upper phase was transferred to a conical test tube (10 × 75 mm) and taken to dryness at 35° with a stream of nitrogen.

Gas-Liquid Chromatography – The esterified sample was mixed with 50 μl of *n*-heptane to effect solution. An aliquot of it was analyzed with a model 402 Hewlett Packard High Efficiency Gas Chromatograph with dual hydrogen flame detectors using glass columns containing various packings (Applied Science Labs., Inc.). Isothermal conditions were maintained and gas flow rates of 25, 35, and 200 ml/min were employed for helium, hydrogen, and air, respectively.

Quantitation – The type and levels of methyl esters prepared from the α-fetoprotein extract were determined by comparing the heights of each component eluting with those of a mixture of standard fatty acids (Applied Science Labs., Inc., and Supelco, Inc.). Differences in extraction and methylation efficiencies of individual fatty acids were corrected for by the recoveries of the *n*-heptadecanoic acid employed as the internal standard. Estimates of precision were obtained by use of a stock solution of albumin to which arachidonic and docosahexaenoic acids had been added. The standard deviation was then calculated from the results of 13 separate experiments. The mean and standard deviations experienced for each of the fatty acids in the stock solution were as follows: 16:0 (1.11 ± 0.08), 18:0 (0.99 ± 0.05), 18:1 (0.74 ± 0.04), 18:2 (0.38 ± 0.02), 20:4 (1.48 ± 0.13), 22:6 (0.95 ± 0.05).

Defatting and Reconstitution of α-Fetoprotein

Preparation of Fatty Acid-free α-Fetoprotein – A modification of the method used by Chen (17) with albumin was employed. Forty-five milligrams of activated charcoal was added to a 15-mg sample of protein in 10 ml of distilled water at 0°. The pH was then carefully adjusted to 3.0 with 0.1 N HCl and the mixture incubated with shaking at 0° for 2 h. The solution was then centrifuged at 25,000 × *g* for 30 min at this temperature. The supernatant containing the lipid-free protein was then decanted from the charcoal and adjusted to pH 7.0 with 0.1 N NaOH.

Addition of Arachidonic Acid to Lipid-free α-Fetoprotein – An amount of the fatty acid sufficient to provide a 3- to 40-fold molar excess over the amount of protein to be employed was dissolved in *n*-heptane and added to a test tube (15 × 125 mm). The solvent was then removed at 35° with a stream of nitrogen. A solution of the lipid-free α-fetoprotein in distilled water was added and the contents allowed to react for 1 h at 22° with gentle shaking.

RESULTS

Identification of Fatty Acids Extracted from α-Fetoprotein – A chromatogram of the methyl esters of the fatty acid fraction which was extracted from α-fetoprotein is shown in Fig. 4. The first five components eluting were identified by comparing their relative retention times with those of a mixture of methyl esters of known fatty acids which eluted as shown in Fig. 4. The identifications of the slower eluting components designated as 20:4 and 22:6 were less certain due to the increased possibility that other fatty acid methyl esters might have identical relative retention times. Since the 20:4 component appeared to be arachidonic acid, preliminary efforts were directed to the identification of the 22:6 material, which at this stage of the experiments was designated as Component X. Preliminary experiments indicated that it was not a bile acid, cholesterol, glyceride, prostaglandin-type material, or estradiol. The latter material was investigated due to the known binding of this substance by α-fetoprotein. Sufficient Component X was isolated by gas chromatography for additional studies. Analysis of ozonolysis products gave no identifiable components. Mass spectrometric analysis was also employed, but no high molecular weight ion species was obtained that proved useful in identification. However, fragmentation ions

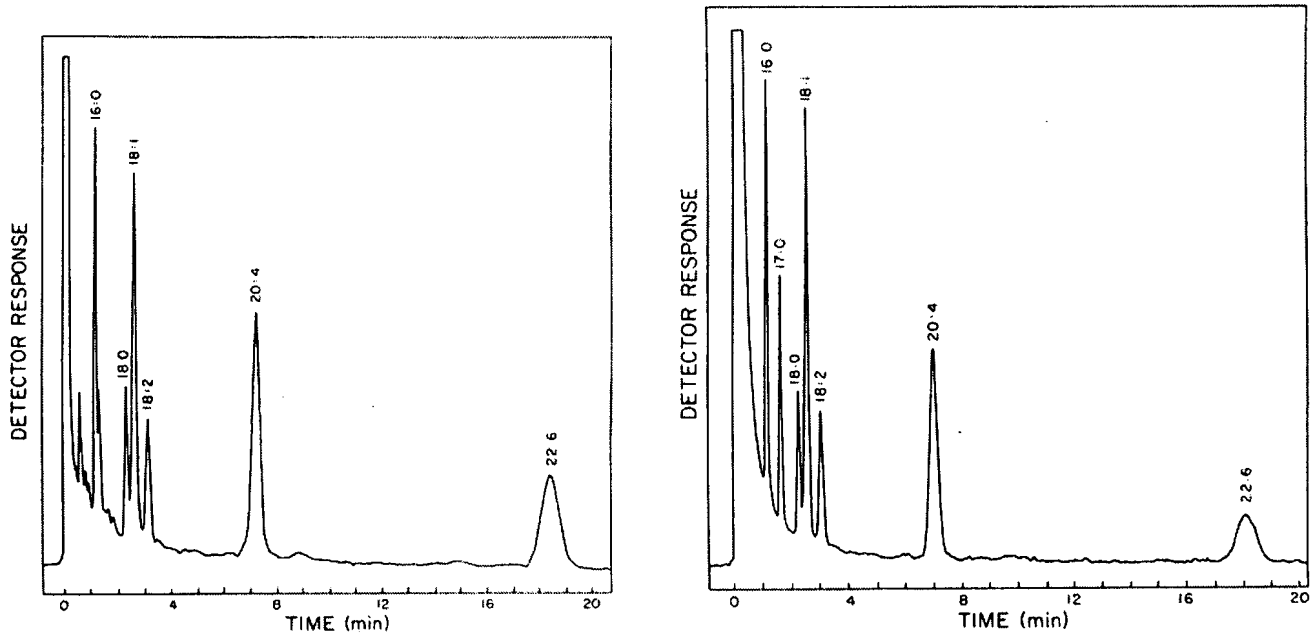


FIG. 4. Chromatogram illustrating the separation of the methyl esters of fatty acids by gas-liquid chromatography on a glass column (0.2 × 175 cm) packed with 3% Silar 5 CP on Gas-chrom Q, 80 to 100 mesh, at a temperature of 185°. The chain length and number of double bonds in each fatty acid are indicated by the numerals located over each peak. *Left*, fatty acid methyl esters of α-fetoprotein; *right*, standard fatty acid methyl esters.

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similar to those seen with samples of methyl arachidonate were noted and suggested that a higher molecular weight unsaturated fatty acid was involved. Chromatography of a series of C-20 and C-22 fatty acids containing various degrees of unsaturation indicated that the compound in question was 4,7,10,13,16,19-docosahexaenoic acid. However, nervonic acid (24:1) was also found to have an identical relative retention time using the conditions shown in Fig. 4. When columns containing 3% OV-1 or 3% OV-225 were employed, the methyl ester of 24:1 was completely resolved from the methyl esters of 20:4 and 22:6. Furthermore when known amounts of the methyl esters of 20:4 and 22:6 were added to the fatty acid methyl esters obtained from α-fetoprotein and then chromatographed on the above types of column packings, each at several temperatures, chromatograms with symmetrical peaks of increased heights for both these components were obtained. Chromatograms illustrating these experiments are shown in Fig. 5. Similar results were obtained at a temperature at 195° and when a 3% OV-225 column at 200° and at 230° was employed.

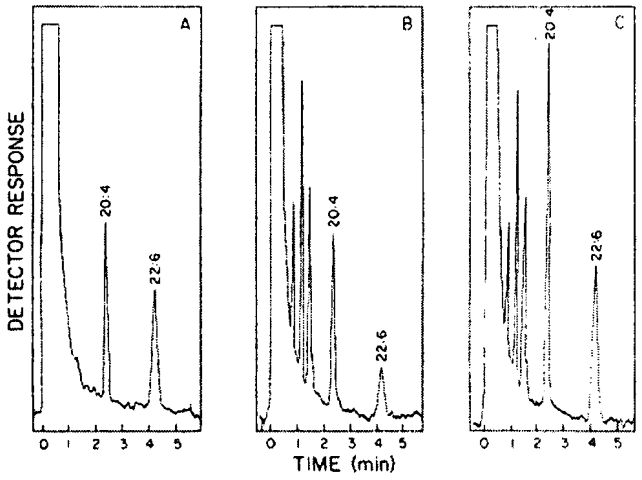


FIG. 5. Chromatograms illustrating the identification of the methyl esters of the 20:4 and 22:6 components of α-fetoprotein. The elution profile of a mixture of the methyl esters of arachidonic acid (20:4) and 4,7,10,13,16,19-docosahexaenoic acid (22:6) is shown in *A*, the methyl esters derived from α-fetoprotein in *B*, and a mixture of the two standards with the esters derived from α-fetoprotein in *C*. The chromatography was performed on a glass column (0.2 × 175 cm) packed with 3% OV-1 on Gas-Chrom Q, 100 to 120 mesh, at a temperature of 225°.

Quantitation of Fatty Acids in α-Fetoprotein—The molar ratios of each fatty acid in the pH 4.7 isoelectric form of α-fetoprotein from three different preparations is shown in Table I. The values range from 2.39 to 3.09 mol of fatty acid/mol of protein and the unsaturated fatty acids account for 86 to 89% of the total. The quantities of the 20:4 and 22:6 components range from 54 to 70% of the total fatty acids and were found to show considerable variation in different preparations. Fetal albumin isolated from the same extract was found to contain 0.7 mol of fatty acid/mol of protein. The unsaturated fatty acids in this material are present in relatively high concentrations, but the 20:4 and 22:6 components comprise only 11% of the total. The fatty acid levels of adult albumin are also shown in Table I. The molar ratio of fatty acids per mol of albumin (2.44) is similar to that of α-fetoprotein, but only 2% of the total is due to 20:4. No 22:6 component was found. The unsaturated fatty acids bound to adult albumin consist mostly of 18:1 and account for only 32% of the total. The pH 5.3 isoelectric point fraction of α-fetoprotein was found to contain less than 0.01 mol of fatty acid/mol of protein.

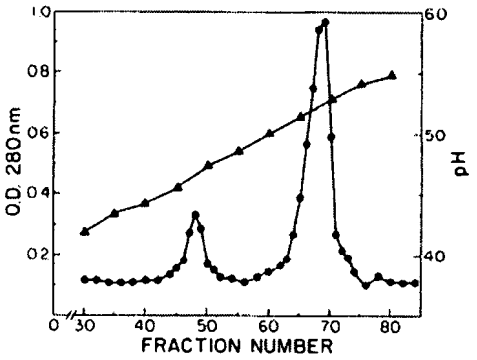


FIG. 6. Result for an isoelectric focusing experiment on 15 mg of the major pI, 4.7 component of purified α-fetoprotein following its treatment with charcoal to remove fatty acids. A pH 4 to 6 Ampholine was employed and fractions of 1 ml were collected (▲——▲, pH; ●——●, optical density at 280 nm).

TABLE I

Concentrations of fatty acids extracted from α-fetoprotein and human serum albumin

Protein[a]	Moles fatty acid/mol protein[b]							Per cent of total fatty acids	
	16:0	18:0	18:1	18:2	20:4	22:6	Total	20:4 + 22:6	All unsaturated
AFP (pI, 4.7 of Fig. 3)	0.21 (8)	0.05 (2)	0.66 (28)	0.17 (7)	0.29 (12)	1.01 (42)	2.39 ± 0.07	54	89
AFP[c] (pI, 4.7)	0.20 (8)	0.14 (5)	0.28 (10)	0.17 (7)	0.99 (37)	0.88 (33)	2.66 ± 0.10	70	87
AFP[c] (pI, 4.7)	0.33 (11)	0.11 (3)	0.51 (16)	0.46 (15)	1.20 (39)	0.49 (16)	3.09 ± 0.11	55	86
HSA[d] (fetal, pI, 4.8)	0.10 (15)	0.02 (3)	0.37 (55)	0.10 (15)	0.05 (7)	0.03 (4)	0.70 ± 0.02	11	81
HSA[e] (adult, pI, 4.8)	0.83 (34)	0.83 (34)	0.48 (20)	0.24 (10)	0.05 (2)	(0.00) (0)	2.44 ± 0.08	2	32

[a] The data in parentheses in this column refer to the source of protein and their isoelectric points.

[b] Based on molecular weights for human serum albumin (HSA) and α-fetoprotein (AFP) of 68,000 and 70,000, respectively. The fatty acid designations are referent to numbers of carbon atoms and double bonds (23). The values in parentheses are the percentages of the total fatty acids. The fatty acids were extracted from the various protein preparations and quantitated as described under "Materials and Methods." All determinations were done in triplicate unless otherwise stated.

[c] Lack of material permitted only a single fatty acid determination for these preparations.

[d] Purified by isoelectric focusing of first component eluting from the Con A-Sepharose affinity chromatography experiment shown in Fig. 2.

[e] This material was purified from serum by chromatography, salt fractionation, and isoelectric focusing techniques.

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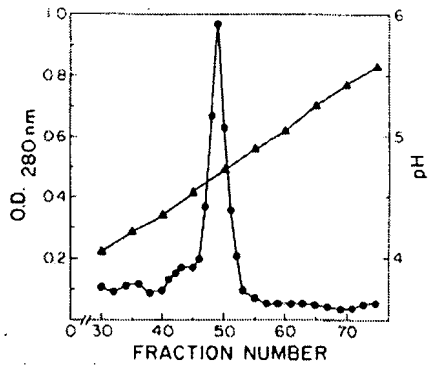


FIG. 7. Result for an isoelectric focusing experiment on 8 mg of lipid-free α-fetoprotein which was reconstituted with arachidonic acid. A pH 4 to 6 Ampholine was employed and fractions of 1 ml were collected (▲——▲, pH; ●——●, optical density at 280 nm).

Removal of Fatty Acids and Reconstitution – The result of isoelectric focusing a sample of α-fetoprotein from which fatty acids were removed by charcoal treatment is shown in Fig. 6. Approximately 84% of the material in the column is the lipid-free component with an isoelectric point of 5.3. More of the residual pI 4.7 material could be converted to the fat-free form by using higher ratios of charcoal to protein, or by longer periods of incubation at pH 3.0, or both. However, these conditions appear to result in decreased yields of total protein. Arachidonic acid was added to the lipid-free fraction in a molar ratio of 40:1 and then subjected to isoelectric focusing. The result shown in Fig. 7 indicates that all of the protein was converted to material with an isoelectric point of 4.7 characteristic of the major component isolated from fetal tissues. Limitation of material to the present time have prevented more extensive investigations of this type.

DISCUSSION

Human α-fetoprotein, separated by the relatively mild method described, binds all of the fatty acids found in the serum albumin of adults and in addition, contains 4,7,10,13,16,19-docosahexaenoic acid (22:6) which is not a normal constituent of the latter protein. The relative proportions of C-16 and C-18 fatty acids in α-fetoprotein agree well with that of human serum albumin as reported by Saifer and Goldman (23), but differ somewhat from those of the human serum albumin analyzed in this work. This discrepancy may reflect differences in purification procedures. Human fetal serum albumin had only about one-third the amount of fatty acids as α-fetoprotein and also has significantly lower amounts of the 20:4 and 22:6 components. The concentration of albumin is from 5 to 7 times greater than α-fetoprotein in fetuses of the age employed in this study (24) which suggests that the latter protein has not only a greater affinity for binding fatty acids in general, but also has a greater specificity for the unsaturated forms than does albumin.

The possibility exists that the 20:4 and 22:6 components of the α-fetoprotein could have been derived from fetal tissues as the result of the homogenization procedure. However, it would be anticipated that the serum albumin, a protein with a high affinity for fatty acids and present at much higher concentration than α-fetoprotein in the fetus, would also have shown the presence of the 22:6 component. The relatively high content of unsaturated fatty acids in α-fetoprotein, particularly of 20:4 and 22:6, may indicate that they serve some specific function in the fetus when combined with this protein.

Isoelectric focusing experiments demonstrated that α-fetoprotein shows considerable heterogeneity. Approximately 84% of it had an isoelectric point of 4.7 while the remaining 16% was isoelectric at 5.3. It is not known if this heterogeneity exists *in vivo* or if it is a consequence of the isolation procedure. The method we employed was relatively mild compared to those of some other workers who have utilized conditions entailing 3 M sodium thiocyanate (9), 8 M urea (25), 1 M acetic acid (26), 15% dioxane at room temperature (27), 4 M guanidine HCl (7), and pH values as low as 1.8 (28) in their isolation procedures. These conditions may modify the protein since the biological activity of mouse α-fetoprotein is altered by even the relatively mild procedure of dialysis against 0.5 M KCl (26). One of our concerns is that the variation in fatty acid contents that we have experienced with preparations of α-fetoprotein may reflect small loses that may be difficult to circumvent. Nevertheless, the question of the relationship of the degree of heterogeneity of α-fetoprotein to the isolation method can not be ascertained at the present time.

The two isoelectric components noted in our α-fetoprotein preparation have been previously observed and it has been suggested that they may reflect differences in sialic acid content (16). However, others have indicated the above forms contain the same amounts of this carbohydrate (29). The results described in the present work on the human protein strongly suggest that the difference between the isoelectric point 4.7 and 5.3 components is due to the presence and absence of fatty acids, respectively. The binding of fatty acids may be co-operative since we find no intermediate forms between the material that lacks fatty acids and the form with an isoelectric point of 4.7 which contains 2 to 3 mol of fatty acid/mol of protein.

Rat and mouse α-fetoprotein have been reported by some investigators to show microheterogeneity (30, 31). These workers, however, do not agree as to whether the variations in sialic acid content are responsible for these differences. It would be interesting to determine whether the various electrophoretic forms of these proteins also relate to variations in fatty acid content.

A considerable interest has recently developed relative to a possible biological role for α-fetoprotein in the mixed lymphocyte test and in the inhibition of lymphocyte transformations induced by mitogens and antiserum to human thymocytes. Some workers have reported that human α-fetoprotein, isolated by different procedures, suppresses human lymphocyte function (29, 32–34) while others indicate an augmentative effect (35). Much of the controversy may relate to the heterogeneity discussed above, since various forms of α-fetoprotein could have different effects. For example, the ability of human α-fetoprotein to suppress the mitogenic response of human lymphocytes has been correlated with the relative amount of the most acidic electrophoretic component present in the serum of patients with hepatocellular carcinoma (29, 34). These workers found that the presence of sialic acid was not necessary for the effects noted, but others have indicated that this component is required for the inhibition of antibody formation in mouse splenic lymphocyte cultures (36). Additional controversy results from α-fetoprotein inhibiting some *in vitro* immune phenomena in mice and rats, but not exhibiting general immunosuppressive functions *in vivo* (9). These types of conflicting results make an interpretation of the biological function of α-fetoprotein difficult. The relatively drastic conditions often employed in the purification of this protein could be related to the inconsistent results and be a

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reflection of variations in its content of fatty acids.

Several observations may indicate that specific fatty acids are important in the biological activity of α-fetoprotein. It is interesting to note that this protein as well as serum albumin derived from human cord blood inhibits various lymphocyte functions, but that albumin of adults was without significant activity (37). The latter protein differs from the others in that it lacks the 22:6 component. Both the 20:4 and 22:6 fatty acids are present in fetal albumin and α-fetoprotein, although we have found that they are present in the latter protein in concentrations 6 and 35 times greater, respectively. Further evidence suggesting that fatty acids may have a functional role relates to the possibility that they increase the binding of estradiol by α-fetoprotein in a manner analogous to their influence on steroid binding by albumin (38). The presence of estrogen in mouse α-fetoprotein has been shown to be necessary for its functional activity in the inhibition of mitogen-induced transformation of lymphocytes (26). However, it is not known whether this relates to the amounts or types of fatty acids bound to the mouse α-fetoprotein. We are presently investigating the importance of fatty acid components of α-fetoprotein in the mixed lymphocyte reaction and on the inhibition of lymphocyte transformation by Con A and phytohemagglutinin. The ability to remove fatty acids from α-fetoprotein and to reconstitute the protein as shown in the present investigation will permit studies of the effects of individual fatty acids or combinations of them on such immunological processes.

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